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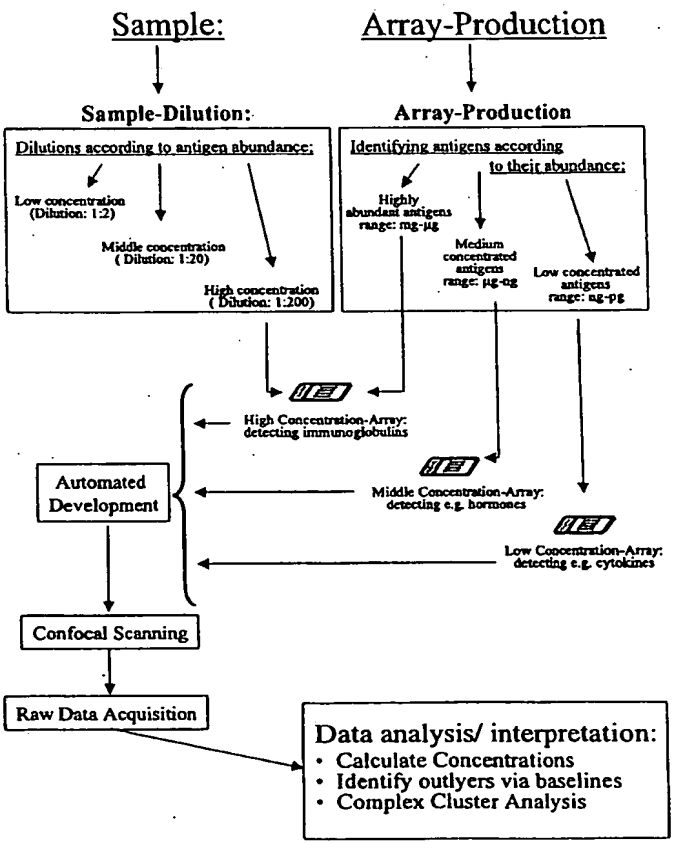
(54) Title: PARALLEL MINIATURIZED QUANTITATIVE IMMUNOASSAYS



(57) Abstract: Protein micro-arrays are disclosed, with methods for their design, production and automated raw data processing, quality control and evaluation, that are suitable for performing parallel miniaturized quantitative binding assays which exhibit improved performance for automated high-throughput and parallel analyses of multiple parameters. The micro-arrays and methods of the invention are particularly well adapted to immunoassay analyses in which antibodies or antigens form the micro-arrays.

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Parallel Miniaturized Quantitative Immunoassays

Field of the Invention

The invention relates to the field of parallel and miniaturized assays arrayed on a solid surface in chip-format, relating particularly to arrays for use in binding reactions and especially to arrays for use in immunoassays used to determine the levels of multiple compounds present in a given sample, the most preferred samples being biological. More particularly, the invention relates to techniques by which a plurality of molecular components of one or more biological samples may be screened in parallel. Most particularly the invention relates to techniques wherein said molecular components are recognized by immune-reactive molecules, as well as to techniques enabling and facilitating efficient screening analyses of multiple samples, especially where a broad dynamic range needs to be covered by the assays, large numbers of samples need to be analysed, where only limited volumes of sample are available and where direct comparability between the data sets generated is of assistance for further use of the results.

Background of the Invention

Binding assays, including receptor-based assays and antibody-based immunoassays are among the most commonly used type of diagnostic assay for the detection and quantitative analysis of biomolecules. The introduction of the competitive binding assay, using radioisotopes, was a major milestone in the development of binding assays, particularly as applied to antibody-based immunoassays (Berson and Yalow, 1959) and later enzyme-labelled immunoassays (Engvall and Perlmann, 1971). At that time, most immunoassays were based on polyclonal antisera drawn from immunized rabbits. This changed with the introduction and development of monoclonal antibodies (Köhler and Milstein, 1975). The major trends in antibody-based diagnostics over the past few decades have been increasing advances in assay specificity, assay

sensitivity, and in detection technologies. This has been achieved in large part by the development of a number of different assay formats. A particularly significant step in assay design was demonstrated by Wide (1971), who first captured antigen using solid-phase-bound antibody and then quantified the amount of captured antigen using a second antibody directed against the antigen and labelled to facilitate quantification of bound material. (The second antibody thereby completed the 'sandwich' of antigen between the two antibodies; at this time each antibody preparation being polyclonal.) More recently, developments have been directed towards miniaturization and parallel assay performance in order to apply immunoassay techniques to high-throughput screening approaches (for example: Huang et al., 2001, De Wildt et al., 2000). Reduction of size offers a number of advantages, for example reduced reagent costs and faster chemical delivery, although maintaining equivalent sensitivity with significantly reduced reaction size requires an improvement in detection technologies.

In the commonly used standard immunoassay format antibody molecules specific for a single antigen are immobilised on the surface of a standard 96-well micro-titre plate within individual reaction wells, where the antibodies remain in a liquid environment. Binding of the antibody molecules is facilitated by specific chemical modifications to the surface of the support. The antibodies immobilized on the surface of the wells of the micro-titre plate are incubated with individual samples or with standard (reference) protein. The size of the wells of a standard 96-well micro-titre plate limits the maximum volume that may be used in such an assay. Following sample incubation antigen concentration is determined, depending on the assay format, by detection of a signal generated to correlate with the amount of antigen in the sample. Primary data are measured in relation to each well of the micro-titre plate in such forms as radioactivity, fluorescence, optical absorption (A) or optical density (OD). There are different types or formats of immunoassay known in the art, which include 'direct', 'sandwich', and 'competitive' immunoassays (J. R. Crowther, 1995).

Nevertheless, although well known in the art, these approaches inherently suffer from major limitations in the design, flexibility and application of the analyses to which

they may be applied. Included in the factors contributing to these limitations are those listed below.

i) Immobilization of the capture antibody in each well of the micro-titre plate requires a significant quantity of this antibody, which may be expensive to obtain or produce, or may be restricted in its availability. These constraints may also apply to other components needed for the assay, for example the labelled standard (reference) protein in the competitive format or the second antigen-specific antibody as used in the 'sandwich' format.

ii) Incubation of sample is performed in each well of the micro-titre plate and thus also needs a significant volume. This is a particular disadvantage when the sample volume available is limited by the biological system being investigated because the number of analyses able to be performed might be insufficient for adequate characterization of the sample. For example, when analysing murine tissues the maximum volume of plasma that can be obtained at a single sampling from an individual mouse on an ongoing basis is 300 µl.

iii) Parallel assays may be performed in an individual reaction well of a standard micro-titre plate if using different fluorescence dyes per parameter, but this approach is limited by space, sensitivity of the respective assays, suitable detection devices and the costs of instrumentation and reagents with differential characteristics of reactivity and detection. In the classic format, where a coloured precipitate or coloured solution is generated via an enzymatic reaction, parallel immunoassays in one well are impossible (ELISA assay; enzyme-linked immunosorbant assay).

Attempts towards further miniaturization have been undertaken in order to circumvent the limitation of the 96-well micro-titre format. Among these, the use of micro-titre plates with 384 or 1528 wells have been successful. More recently the first array-based protocols became known in the art: Lueking *et al.*, (1999), Mendoza *et al.* (1999), Silzel *et al.*, (1998).

In recent years parallel approaches to expression profiling of cellular mRNA-levels, based upon sample hybridization to immobilized DNA, have been introduced

into the art. These approaches employ micro-array technology to generate a large quantity of information relating to a given biological sample. However, DNA-based protocols are restricted in at least two aspects which limit their direct adaptation to miniaturized immunoassay analyses:

1. There is a limitation with respect to the dynamic range of the micro-array-based protocols currently known in the art. This is sufficient for gene expression analyses, using immobilized DNA, in which ratios of green and red fluorescence are generated which are in most cases below 10, and often below 3 or 4. The wide range of protein concentrations encountered in many biological samples, encompassing more than eight orders of magnitude, is generally far beyond the dynamic range available in current micro-array-based approaches.

2. To date there is no labelling method available that guarantees constant proportional labelling of proteins in biological fluids in a defined manner, for instance one fluorescence-dye per given protein. Therefore, direct labelling of control versus sample with two different dyes as performed in DNA-based or RNA-based approaches and calculating expression-ratios does not currently provide reliable quantification.

Parallel and miniaturized analysis of antigens in a biological sample to gain maximum information, especially when only small volumes of sample are available, is highly desirable in research using small animals or limited amounts of human or animal tissue. At present, however, numerous practical difficulties prevent the implementation of such parallel and miniaturized formats, including inconsistencies in protein binding, intra-chip and inter-chip variability, and the narrow dynamic range and data dispersion that are characteristic of such analyses.

Summary of the Invention

The present invention provides miniaturized protein binding assays, preferred embodiments offering protein micro-arrays of a specified design and methods for the production of such micro-arrays, particularly where the protein immobilized in the micro-array format comprises at least one antibody or antibody fragment.

The method of producing protein micro-arrays provided by the invention obviates the thin layer or membrane coating the surface of the micro-array support that is required to maintain binding characteristics of arrayed proteins by certain techniques recently contributed to the art. As the invention provides for the micro-array support to bind directly the protein forming the array, the support is also termed a 'substrate'.

The present invention addresses problems regarding standardization of the array measurements or experiments, automated raw data processing and quality control procedures and offers solutions for an improved performance of antibody-arrays used in a high-throughput format for the analysis of multiple parameters, especially in analysing biological fluids, or in samples containing solutions of biological materials. A preferred embodiment of the invention employs reporter molecules that themselves comprise proteins, in order that these proteinaceous reporter molecules may be satisfactorily immobilized on the substrate surface (preferably by the immobilization method of the invention) to provide additional control features.

The invention is particularly useful when only small volumes of samples are available, as where research or diagnostic procedures utilise tissues that are available only in limited amounts, *e.g.* those from human or animal sources – particularly small animals such as mice – in forensic detection or in research involving slow-growing tissue cultures or slow-growing plant tissues. The invention provides improved chip design, suggests ways to broaden the dynamic range of sample analysis and optimal data normalization using internal and external controls. In addition, an automated data management pipeline is introduced, which allows: (i) processing of raw data; (ii) extensive analysis and control of the reliability of the individual assays; and (iii)

evaluation of large data sets, thereby generating maximal information from the samples under analysis. The latter aspect is particularly advantageous in the analysis of biological samples, enabling (for example) the results obtained to be correlated with parallel series of physiological data or enabling them to provide information about the relevant metabolic context.

The present invention provides the novel and inventive miniaturisation and parallel arrangement of immunoassay components as well as specific control features to ensure the optimal data evaluation and allow comparison of results generated in independent experiments. The invention provides simultaneous identification and quantification of multiple molecular species with capability of binding to a capture protein (*e.g.* ligand with receptor, immune reactive molecular species with antibody) on a miniature scale, with a sensitivity covering a wide concentration range. In further embodiments of the invention large numbers of binding assays, preferably being immunoassays, capable of detection and quantification of sample over a wide concentration range, which are preferably arranged in micro-arrays as represented by the spots of the capture binding protein on the substrate surface, are subjected to automated parallel analysis in a 'high-throughput' manner. In further preferred embodiments of the invention said large numbers of binding assays are from 250 to 1000 binding assays. Said wide concentration range over which sample is quantifiable is, in preferred embodiments of the invention, at least a factor of 10^6 (typical examples being from picograms to milligrams of a particular protein per millilitre of original sample. Such binding assays of the invention, including immunoassays, are capable of detecting the variety of protein parameters within the range that such molecules are generally encountered in biological fluids, and also other ligands and antigenic molecules within the range of concentrations generally encountered in biological fluids, such as hormones at 100 nM to 100 μM. In more preferred embodiments of the invention said wide concentration range over which sample is quantifiable is, in preferred embodiments of the invention, at least a factor of 10^9.

In a preferred embodiment of the invention, the parallel miniaturized quantitative immunoassays of the invention generate sets of data from a series of

samples: in a particularly preferred embodiment these samples being biological fluids from test animals that have previously undergone a defined treatment. The invention facilitates analyses of large sets of such samples (for example from animals or humans) in order to obtain data rapidly from a complex arrangement of experiments or from screening projects.

Preferred embodiments of the invention enable the analysis of a broad range of parameters and concentration ranges that have clinical relevance (diagnostic applications) or having potential clinical relevance (research applications), in order to provide detection or characterisation of disease states in test organisms. In especially preferred embodiments the test organisms are animals, being mammals in the most preferred embodiments. Diagnostic applications may be directed to humans or to domestic or laboratory non-human animals, especially rodents. Research applications may be similarly directed. In a particularly preferred embodiment the invention is applied to analysis of samples from small test animals such as mice that are able to supply such biological samples as blood or solid tissues only in limited amounts, particularly on an on-going basis.

To ensure comparability and enable standardisation between data sets generated at different times, or with different arrays (or chips) or by different sub-arrays on a single chip, preferred embodiments of the invention include arrays which are designed with a number of control features that may be used to correct for variations in assay performance, inter-chip and intra-chip variability. These features are essential for a controlled and reproducible high-throughput use of antibody-arrays.

The invention provides at least semi-quantitative estimation of the concentration of individual immune-reactive molecular species within a particular sample (*i.e.* accuracy within one order of magnitude and reproducibility within ±50%) by comparison with concentrations of standard, equivalent molecular species. Preferred embodiments of the invention provide quantitative estimation of such concentrations (*i.e.* accuracy within ±50% and reproducibility within ±20%). More preferred embodiments of the invention provide greater accuracy and reproducibility, *e.g.* an

embodiment providing accuracy within ±10% and reproducibility within ±5% is preferred to an embodiment employing the equivalent estimation and providing accuracy within ±20% and reproducibility within ±10%. In preferred embodiments of the invention these individual immune-reactive molecular species detected within a particular sample, and the corresponding standard, equivalent molecular species, are proteins or may be other forms of molecule bearing recognizable epitopes, such as peptides, nucleic acid sequences or small molecules. The invention is capable of employing internal and external standards independently, as required.

Brief Description of the Figures.

Figure 1 depicts a preferred assay procedure for use of the parallel, miniaturized and automated immunoassay platform of the invention. Specific antibodies are printed on a substrate forming a two dimensional array. Parameters are combined according to their abundance in the biological sample. Dilutions of the biological sample are performed to meet the dynamic range of the respective immunoassay. During or following the assay, data acquisition, analysis and interpretation is performed.

Figure 2 schematically outlines a typical, preferred design for a single array and control features therein. The design of the array as applied to analysis of particular murine plasma samples: capture proteins are antibodies printed in three different concentrations; detection controls (in this case detection antibodies used in the assays to be performed with the particular array, these being secondary antibodies labelled with one or more reporter molecules) in three different concentrations. Internal control features are designated 'IC' and include (i) an anchor row which contains reporter molecules employed in the assays for which the micro-array is to be used, integrated into each array, and (ii) a row of a capture protein unrelated to the assays to be performed (in this case anti-gp41 antibody as a non-murine parameter) for determining intra-chip and inter-chip reporting efficiency. (See Example 1.)

Figure 3 depicts a scanned image of the resulting spot morphology and spot stability of different printing buffers as listed in **Table 1**. Streptavidine-Cy™3-conjugate (diluted 1:500 in PBS) and Streptavidine-Cy™5-conjugate (diluted 1:1000 in PBS) were mixed and added to the printing buffer. Confocal slide images (**A**) scanned directly after the printing process and (**B**) after blocking in PBS containing 1% BSA and three washes with PBS.

Figure 4 depicts the performance of parallel, miniaturized and automated immunoassays of the invention and that of standard immunoassays: standard curves generated in parallel for four different immunoglobulins are compared. Relative signals for four individual proteins (Fig. 4A: IgA; Fig. 4B: IgG1; Fig. 4C: IgG2a; Fig. 4D: IgG3) were plotted against the concentration of standard proteins displaying a comparable assay dynamic of the antibody microarrays and conventional ELISA assays.

Figure 5 depicts graphical charts of standard curves of sandwich immunoassays and competitive immunoassays generated by using at least four different concentrations of standard protein, a blank for each parameter and one slide per concentration. Incubation with standard proteins was performed for 2 hours at room temperature. Best-fit curves were calculated and used as quality control for assay performance. With a coefficient of correlation of less than 0.89 an immunoassay would be regarded as failed. Fig. 5A depicts representative graphs of standard curves of the cytokines TNFα and IFNγ determined using the sandwich immunoassay format. Fig. 5B depicts representative graphs of standard curves for immunoglobulins IgG2a and IgG3 as determined by competitive immunoassay format. R: coefficient of regression.

Figure 6 depicts graphical charts of samples as measured in high throughput screening of mouse plasma samples. Fig. 6A depicts IgA-concentration as determined in mouse plasma collected from individual animals. Results in the graph are combined from three independent experiments. Fig. 6B depicts luteinizing hormone-concentration as measured in mouse plasma collected from individual animals. Results are combined from two independent experiments.

Figure 7 schematically outlines raw data processing and quality control procedure. The data analysis is divided in two independent branches, which both delivers the processed and quality checked data to the central data depository. In order to calculate distinct protein concentrations the standard curve is used to calculate the concentration values after a background reduction and normalizatio. In contrast, for parameters which are provided in relative units, the median of a large set of samples is calculated and used for normalization purposes. In both cases the standard curve and the respective correlation coefficient are used as automatic quality control parameters. In addition the reliability of the assays and processed data is routinely monitored by a visual inspection.

Figure 8 depicts a set of immunoglobuline data generated during the routine use of the platform described in the invention was processed follwing the automated dataprocessing pipeline. Fig. 8A. represents processed a raw data set transformed into distinct analyte concentration. Fig. 8B represents immunoglobuline raw data (IgG1, IgM) transformed into relative analyte concentration. This analysis method is of particular importance to identify outlyers with extraordinarily high or low analyte levels.

Table 1 summarizes components of a selection of preferred printing buffers.

Detailed Description of the Invention

The invention provides methods for the detection and quantification of molecules recognized by immune-reactive molecules in a highly parallel and miniaturized manner. In these methods novel and inventive control features overcome the limitations of known protein-based micro-array chip-technologies with respect to inter-chip and intra-chip variability and inconsistent spot morphology. Furthermore, by greatly enhancing the small dynamic range of assays based upon micro-array chip-technologies relative to standard microtitre plate techniques, the invention makes micro-array chip-technologies compatible with the requirements for complex and comprehensive sample analysis. In

order to characterize a large set of samples in a high-throughput manner the invention comprises a technical design which allows a parallel and simultaneous analysis of numerous arrays, where each array contains a variable number of individual features characterizing the individual biological sample (Fig. 1). The invention can be used either in a 'sandwich' immunoassay format, or direct immunoassay format, or competitive immunoassay format. The performance of the method is greatly enhanced with respect to reproducibility, standardization and number of analyses by the integration of automated liquid handling procedures.

Definitions

The term 'chip' describes the device used to carry out the analysis of samples, comprising the substrate and the protein-array or protein–arrays deposited onto and attached to the substrate surface by, for example, printing or spotting techniques. Examples of samples include, but are not limited to biological fluids and extracts of biological material, tissues and organs from such sources as plants, micro-organisms, animals and humans. Examples of biological fluids are described below.

The term 'substrate' is used for the solid support onto which the protein solution or protein solutions are delivered in order to create the array of protein spots. Polypeptides, oligopeptides and peptides are also considered to be 'proteins' in the context of 'protein' micro-arrays, the production of spots for such arrays and the protein-containing solution or solutions used to generate such spots. Preferred supports are of glass or plastic, and generally the surface of the support can be chemically modified.

The term 'spot' is applied to each individual area of substrate surface over which the protein arrayed on the substrate surface is permanently distributed after the protein is delivered to the substrate surface as a protein solution in printing buffer, and dried thereon. Preferred proteins are antibodies (arrays for 'sandwich' and competitive format immunoassays) and antigens (arrays for direct immunoassays). Polypeptides, oligopeptides and peptides are also considered to be 'proteins' in the context of

'protein' micro-arrays, the production of spots for such arrays and the protein-containing solution or solutions used to generate such spots. The spot may be of any geometrical shape or may be irregular shaped. Preferably the diameter of one spot is 100-200 µm and the volume applied to the substrate surface 0,2 nl.

An 'array' is an arrangement of spots in a pattern on a substrate, wherein the spots comprise protein entities – within which term is included proteins, polypeptides and peptides – which are preferably antibodies. Although the pattern is typically a two-dimensional pattern, it may also be a three-dimensional pattern. There may be several arrays on one substrate. In the context of the present invention, a protein micro-array is an 'array' formed on a miniature scale, wherein the term 'protein' refers to said protein entities and may include proteins, polypeptides and/or peptides – in a preferred embodiment being antibodies.

A 'block' is the spot pattern printed by an individual deposition or spraying source (*e.g.* one pin, split pin, or piezo device) onto the substrate surface.

The terms 'printing' and 'printing process' refer to the production of one or several arrays on a variable number of substrates by delivering equal amounts of protein solution onto the surface of the respective substrates. In preferred embodiments of the invention the protein in said protein solution is an antibody or fragments thereof; the protein may be an antigen in addition to, or instead of being an antibody. The printing process is performed by robotic devices, of which particular examples are termed Microarrayer or BioChip Arrayer, that may either use non-contact dispensing or deposition of the protein solution onto the substrate surface (*e.g.* PiezoTipTM-technology (Packard BioChip Technologies, Meriden, USA)), or a method of dispensing or deposition during which the device does contact the surface (*e.g.* split-pin systems (Biorobotics, Cambridge, UK) or pin-and-ring-systems (MWG, Ebersberg, Germany).

The term 'antibody' means an immunoglobulin, whether natural or partially or wholly synthetically produced. The term also includes all derivatives or fragments

thereof that maintain specific binding ability. The term also covers any protein containing a binding domain that is homologous or largely homologous to an immunoglobulin-binding domain. An antibody may be monoclonal or polyclonal. The antibody may be of any immunoglobulin class. Purified derivatives of the IgG class are preferred in the present invention.

The term 'parameter' describes the individual molecular species that an array (or micro-array) is designed to detect or quantify when said array (or micro-array) is used to perform a corresponding binding assay or immunoassay. Preferred embodiments of the invention provide arrays and associated methods designed for simultaneous quantification of multiple parameters in an individual sample by, for example, incorporating a multiplicity of identified capture proteins and/or protein ligands into the array at known locations. Generally each spot would comprise only a single capture protein or protein ligand, but the invention also includes arrays and methods in which a plurality of capture proteins and/or protein ligands are incorporated into certain spots, different reporter molecules being used in conjunction with the assay of each parameter in order to distinguish between the individual results.

The term 'binding assay' describes an adsorbent assay, which in preferred embodiments is enzyme-linked or fluorescence-linked, wherein:

(a) a capture protein is bound onto a substrate ('sandwich' format; competitive format) and incubated with:

(i) aliquots of sample and in separate, comparable reactions, known quantities of "standard" ('sandwich' format); or

(ii) aliquots of sample comprising labelled "standard" in defined concentration (competitive format);

wherein the "standard" represents the ligand, *i.e.* the molecule that binds to the capture protein, and is the parameter measured by the binding assay.

(b) a protein ligand is bound onto a substrate (direct format; competitive format) and incubated with:

(i) aliquots of sample and aliquots of "standard" in separate, comparable reactions (direct format); or

(ii) aliquots of sample comprising labelled "standard" in defined concentration (competitive format);

wherein the "standard" represents the molecule that binds to the ligand, and is the parameter measured by the binding assay.

Preferred embodiments of the invention employ such binding assays for quantification of receptors or adhesion molecules, in each case the ligand being chosen to correspond to the receptor or adhesion molecule of interest. The sandwich and competitive formats use a specific labelled detection molecule (in preferred embodiments a protein, and in particularly preferred embodiments an antibody) that carries a reporter molecule, while in the competitive format the sample-associated reduction of signal obtained from a known concentration of a respective standard molecule labelled with a reporter molecule is measured.

The term 'immunoassay' describes an immune adsorbent assay, which in preferred embodiments is enzyme-linked or fluorescence-linked, wherein:

(a) a capture antibody is bound onto a substrate ('sandwich' format; competitive format) and incubated with:

(i) aliquots of sample and in separate, comparable reactions, known quantities of "standard" ('sandwich' format); or

(ii) aliquots of sample comprising labelled "standard" in a range of known concentrations (competitive format);

wherein the "standard" represents the antigen, *i.e.* the antigen that binds to the capture antibody, and is the parameter measured by the immunoassay.

(b) a protein antigen is bound onto a substrate (direct format; competitive format) and incubated with:

(i) aliquots of sample and aliquots of "standard" in separate, comparable reactions (direct format); or

(ii) aliquots of sample comprising labelled "standard" in a range of known concentrations (competitive format);

wherein the "standard" represents the antibody that binds to the antigen, and is the parameter measured by the immunoassay.

Preferred embodiments of the invention employ such immunoassays for quantification of antigens and of antibodies and also for quantification of receptors or adhesion molecules, in each case the antibody being directed to an epitope characteristic of the antigen, antibody, receptor or adhesion molecule of interest. The sandwich and competitive formats use a specific labelled detection molecule (in preferred embodiments a protein, and in particularly preferred embodiments an antibody) that carries a reporter molecule, while in the competitive format the sample-associated reduction of signal obtained from a known concentration of a respective standard molecule labelled with a reporter molecule is measured.

The term 'immunoassay' describes an immune adsorbent assay, which in preferred embodiments is enzyme-linked or fluorescence-linked, where either a capture antibody ('sandwich' format, competitive format) or an antigen (direct format) is bound onto a substrate and incubated with sample or standard protein ('sandwich' format, direct format) or labelled standard protein in defined concentration is mixed with the sample (competitive format) and incubated. Preferred embodiments of the invention employ such immunoassays for quantification of antigens and of antibodies and also for quantification of receptors or adhesion molecules, in each case the antibody being directed to an epitope characteristic of the antigen, antibody, receptor or adhesion molecule of interest. The sandwich and competitive formats use a specific labelled detection antibody that carries a reporter molecule, while in the competitive format the loss of signal of a known concentration of a respective standard protein labelled with a reporter molecule is detected.

The term 'reporter molecule' refers to any molecule that is capable of producing an identifiable or detectable result. Preferred reporter molecules include but are not limited to radiochemicals, fluorescent compounds (*e.g.* rhodamine, DIG, Cy5 (indodicarbocyanin) and Cy3 (indocarbocyanin)), biotin, functional enzymes (*e.g.* alkaline phosphatase, horseradish peroxidase, the *Esherichia coli* ß-galactosidase, the firefly luciferase protein and the green fluorescence protein (GenBank Accession No.

U55762)). In the present invention the use of Cy5 and Cy3 as reporter molecules is preferred.

The term 'ligand' denotes a reactive molecule that binds to a protein arrayed in the micro-array (*e.g.* a capture antibody) or which is itself arrayed in the micro-array and is bound by a detection molecule. Preferred ligands are antigens, preferred detection molecules are antibodies, which may themselves be labelled with a reporter molecule. Ligands may be peptides, polypeptides or proteins, and may be arrayed on a substrate in order to bind and estimate the abundance of the corresponding receptor molecule or binding protein in a sample. The sample is a biological sample in preferred embodiments of the invention.

The term 'biological sample' describes any soluble substance extracted, excreted, or secreted from an organism or tissue of an organism. Samples of particular relevance to the present invention include but are not limited to whole blood, serum, plasma, urine, sinovial fluid, cerebrospinal fluid, tissue extracts, organ extracts and cell extracts.

1. Production of the arrays

One aspect of the present invention provides an array on a substrate for quantification of a given number of ligands in one biological sample. A preferred, standardized size for such an array is 2.5 cm x 7.5 cm, and antigens are preferred ligands. Each array consists of a large set of individual protein spots, each of which comprises protein with specific ligand-binding characteristics. Preferably these protein spots comprise monoclonal or polyclonal antibodies, derivatives of antibodies or antibody analogues, thereby enabling the quantitative analysis of the the ligandfor which the printed antibody is specific.

In addition, each array contains a set of control features (reporter molecule coupled binding proteins, preferably antibodies, for example a monoclonal antibody directed to an epitope unrelated to the sample being analysed) in order to monitor assay-performance, inter-chip and intra-chip variability, the homogeneity of the individual

spots, and to allow a comparison of individual assays performed at different time points (Fig. 2).

Assay performance of a set of protein micro-array-carrying substrates is monitored by integration of standard curves on a plurality of slides of the chip series used in one experiment for each parameter determined in the respective binding assay, which is preferably an immunoassay. In preferred embodiments the protein arrayed on the substrate is an antibody; the protein arrayed on the substrate may also be an antigen. In a further preferred embodiment generation of standard curves is performed on multiple slides of the chip series used in one experiment for each parameter determined. In a particularly preferred embodiment integration of standard curves is performed on eight slides of the chip series used in one experiment for each parameter determined. One chip series used in one experiment is preferably printed on one day, optionally using one master 384-well micro-titre plate as a convenient storage receptacle and source for the antibody-solutions printed. In addition, detection antibodies of each parameter coupled to reporter molecules are spotted as a control for the detection reaction.

Inter-chip variability is monitored by an unrelated immunoassay integrated into each individual block of each array. In a typical forward assay as applied to the present invention a 'distinct species' antigen is added to the samples and the standard protein mixture used for the standard curves at a constant concentration. A 'distinct species' antigen is an antigen obtained from a species distinct from each of the species from which are derived the samples to be analysed using the micro-array. A sandwich immunoassay, where a second antibody specific for the 'distinct species' antigen, labelled with one or more reporter molecules, is used for producing a signal representing the amount of bound antigen and the resulting intensities after performing the detection procedure are used to calculate a normalization factor. The results may either be qualitative, by simple observation of the visible signal produced by the reporter molecule, or may be quantified by comparing with a control sample containing known amounts of protein. In a preferred embodiment of the invention, gp41, a HIV-1 transmembrane protein, is used as unrelated antigen. The most commonly used reporter

molecules are enzymes, fluorophores, or radioisotopes, bioluminescent and chemiluminescent molecules.

Those skilled in the art are aware that the binding in any immunoassay is susceptible to variation from at least two sources:

i) variable amounts of antigen bound to the specific capture antibody;

ii) differential association and dissociation constants of antibodies for their specific antigens in the binding reaction.

A preferred embodiment of the present invention provides for the correction of concentration-dependent variation in signal intensity at the stage of primary capture antibody. This is achieved by labelling antibodies prior to printing onto the substrate surface with one or more reporter molecules. Using a two-colour code, with the capture antibody labelled with one dye, and the detection antibody with a second dye, it is possible to relate the amount of capture antibody printed to the amount of antigen bound.

Integrating an unrelated immunoassay in every block of the array is also useful to monitor intra-array variability, which means the variability between the spots of one array printed by different components of the depositing or dispensing apparatus (*i.e.* by individual split-pins or other corresponding printing devices of the apparatus). In a preferred embodiment of the invention four split pins are used to print one block of rows each. In each block one row of unrelated control capture protein is integrated (*e.g.* anti-gp41 antibody) and used as an unrelated antibody control parameter. In addition, an analysis of the intensities of the signal from individual spots of one particular parameter is performed. The average signal obtained from the spots of one parameter is calculated and the standard deviation determined. Each individual spot intensity is calculated against the average and is excluded if it cannot be distinguished from the background with a pre-determined confidence level (such data are assigned a so-called 'error flag'). In a preferred embodiment of the invention this predetermined confidence level is set at 95%. The variation may differ between the parameters included in one array.

Homogeneity of the spots is monitored by 'anchor spots': an internal control feature of the array, in which reporter molecules are immobilized on the substrate surface to provide an internal control of the efficacy of the reporter molecule signal, its consistency when reproduced within the array, and its consistency from array to array, and chip to chip, allowing historical comparisons and standardizations to be made. In preferred embodiments of the invention, in which the spots of the array are arranged in a regular two-dimensional matrix pattern of rows and columns, the anchor spots form an entire row across the breadth of the array: the 'anchor row' (Fig. 2). In a further preferred embodiment of the invention the reporter molecules of the anchor spots comprise proteins, in order that these proteinaceous reporter molecules may be satisfactorily immobilized on the substrate surface to provide additional control features. The immobilization of the proteinaceous reporter molecule on the surface of the substrate is preferably performed by the protein immobilization method of the invention, which assists in the maintenance of protein conformation during the immobilization drying of the protein on the substrate surface. Preferred reporter molecules for use in the anchor spots are Cy5 or Cy3, which comprise the streptavidin protein (Amersham Pharmacia Biotechnology, Freiburg, Germany). Since with the split-pin printing each pin delivers an individual spot morphology, comparability between two sets of experiments is greatly increased if these differences are taken into account. Each individual spot is present in a set of replicas in order to generate mean data sets further increasing the assay performance and allowing statistical data handling.

In a preferred embodiment of the invention each array comprises a constant number of blocks resulting from the production procedure, for example, four blocks printed by four split pins. Each block comprises a constant number of rows of individual spots with a constant number of replicates containing (i) the primary (capture) antibodies in at least two different concentrations characterizing the individual parameters, (ii) antibody labelled with a reporter molecule, and (iii) in the sandwich immunoassay format detection antibodies of the respective parameter in one or two different concentrations. In a preferred embodiment of the invention 11 rows featuring 11 replica spots per row are printed and the anchor spots contain an antibody that is a

Cy5™-conjugate (Dianova, Hamburg, Germany). The various concentrations of capture antibody broaden the dynamic range of the chip (Fig. 2).

The printed detection antibodies serve as controls for the detection step of the sandwich immunoassay. In a preferred embodiment of the invention the detection antibodies are biotin-conjugates and the signal is produced by an incubation of the array using a streptavidin-Cy3-conjugate. Resulting intensities of the detection antibody rows can be used for normalization of split pin variations resulting in different spot morphologies between the individual blocks of one array and between blocks of different arrays on a different or the same slide. The dye-labelled antibody serves as quality control for the printing process, for normalization of split pin variations between blocks in competitive and direct immunoassays, and can be used as an anchor for the pattern recognition in raw data acquisition by laser scanning. In a preferred embodiment Gene Pix Pro 4000 Microarray scanner plus its Microarray Acquisition and Analysis Software (Axon Instruments, Inc.) is used for data acquisition and analysis following the immunoassay protocol.

The antibody arrays are produced using microarrayers based on printing system (e.g. split pins) or piezoelectric systems or pin-and-ring technology. In a preferred embodiment a microarrayer from Biorobotics (Microgrid II) with split pins is used.

2. Preparation of substrate surfaces

Substrates comprising one or more of a variety of materials can be used to provide the surface for the micro-arrays of the invention (*e.g.* glass, plastic – for example polyethylene, polypropylene, polystyrene, and also substrates having the surface upon which the protein array is deposited being membrane-covered, or covered with a thin film). Of these, the preferred substrate is glass, convenient and inexpensive formats being provided by several manufacturers in the form of glass slides for microscopy, the composition of the glass being accurately defined and optically flat surfaces being available (a preferred option). In a preferred embodiment of the invention substrates containing covalent aldehyde or carboxyl groups are used. The carboxyl groups may be generated by oxidizing aldehyde groups presented on a

substrate surface by incubation with potassium permanganate solution at 1 M in sodium phosphate solution (1,25 M, pH 4,4) for 10 minutes at room temperature with agitation (Abiko et al., 1986). The resulting carboxyl groups are activated with EDC and Sulfo-NHS shortly before coupling of antibodies to the surface (Staros, *et al.*, 1986; Gabarek *et al.*, 1990).

3. Immobilization of capture protein

In order to guarantee an optimal assay performance it is imperative to ensure the ligand-binding functionality of arrayed protein molecules is retained, in spite of their immobilization during array production, until use of the protein micro-arrays in the corresponding assay procedure. In preferred embodiments of the invention the immobilized, arrayed proteins whose binding functionality needs to be retained are the primary protein (primary antibody's ligand-binding functionality to be retained) and secondary antibody (detection antibody's target-binding functionality to be retained) – *i.e.* retention of epitope binding characteristics is of critical importance. The invention comprises a combination of support surface and production buffer in which the protein molecules are printed onto the surface of the substrate. The production buffer is composed such that it is able to:

i. keep the protein molecules that are to form the array fully functional prior to spotting and adherence to the substrate surface;
ii. allow an optimal array production during printing, either with contact printing or ink-jet technologies;
iii. facilitate stable binding of the protein molecules onto the surface after printing; and
iv. prevent the spotted and bound proteins on the solid support from denaturing and to maintain their fully functional conformation.

In preferred embodiments of the invention the protein molecules printed onto the surface of the substrate are antibodies, and the invention provides production buffers that optimise these characteristics in relation to antibody arrays.

Several printing buffers have been validated to meet these needs (Fig. 3, Tab.1). The solid support onto which the arrays are printed is adapted to the buffer in order to support the binding of the antibody, to keep the antibody molecules functional and meet the requirements of raw data acquisition. In a preferred embodiment of the invention the printing buffer and surface have been developed in accordance with the foregoing criteria and to be mutually compatible: stable attachment of proteins is provided by applying phosphate buffered saline (PBS) supplemented with 0,1%(w/v) SDS (sodium dodecyl sulphate) and 5%(v/v) glycerol as printing buffer and using glass slides containing aldehyde groups as a substrate (CSS-100, CEL Associates Inc., USA; SAS, ArrayIt TeleChem., USA). Alternatively, glass slides containing activated carboxyl groups are produced by oxidizing aldehyde group-containing slides.

The SDS decreases the surface tension of the printing buffer, achieving a homogeneous distribution of the antibody-solution in the spot area; the glycerol decreases and slows down the evaporation of the liquid in the printed spot facilitating an optimal and functional binding of the proteins to the substrate. Different printing systems may generate optimal results with variation of the component concentrations of the recommended printing buffer: for example, with the pin and ring technology glycerol concentrations up to 40% can be used, a concentration incompatible with split pins.

4. Composition of chips

The chip platform used in the invention is composed of a set of independent arrays or for high through put screening of one array. In a preferred embodiment of the invention, three independent arrays per slide are printed. The arrays are arranged on the solid substrate in a way that:

i. the arrays can be incubated with one sample or with different samples in parallel;
ii. the arrays can be incubated with different sets of secondary antibodies;
iii. the arrays can be processed simultaneously by a mechanised liquid handler; and
iv. different sample dilutions can be used on individual arrays on an individual substrate.

5. Chip architecture

In order to quantify a broad spectrum of ligands, *e.g.* antigens, in samples adequately the individual analyses should be responsive over a wide dynamic range. As the dynamic range of chip experiments is limited but the concentration of proteins in biological samples such as body fluids ranges over six orders of magnitude a composite approach was developed and implemented. On the individual chip different arrays are printed, each directed to the characterization of a set of multiple binding compounds, which are preferably immune-reactive compounds. Preferably, in each array on the solid support the spots comprising capture proteins (preferably primary antibodies) are grouped in order that the parameters that their respective assays (*i.e.* the ligands of the capture proteins; preferably antigens) generate detectable signal intensities when present in similar concentration ranges within the given set of samples. Naturally, this will be more readily predictable when the micro-array technology is applied to screening procedures and multiple comparable data sets are generated and available for comparison. In order to obtain signal intensities within the dynamic range adapted to the expected protein concentration the relevant concentration of capture protein (preferably primary antibody), sample, detection antibodies as well as labelled standard protein concentrations in case of a competitive assay format (for details see below) were adjusted on the individual arrays.

In a preferred embodiment in which the chips are used for the characterization of blood samples from animals, a chip contains three individual arrays in which the concentration of capture protein in the spots of the array binds an appropriate amount of ligand for the assay to generate a signal that is detectable in proportion across a concentration range of the sample parameter in the range of picograms per ml to milligrams per ml for many proteins. More preferred embodiments extend this range further (Fig. 1). In arrays prepared for detection and quantification of "high-concentration" sample components, capture proteins are deposited appropriately for the detection and quantification of protein abundancies in the range ($\mu g\ ml^{-1}$ to $mg\ ml^{-1}$ range for many proteins, *i.e.* equivalent to the abundancy of immunoglobulins in serum or plasma). In arrays prepared for detection of "medium-concentration" sample

components, capture proteins are deposited appropriately for the detection and quantification of sample components present in moderate abundance (detection range: ng ml^{-1} to µg ml^{-1} for many proteins), such arrays being capable of addressing and quantifying various blood parameters, for example cholesterol, insulin and leptin. In arrays prepared for detection and quantification of "low-concentration" sample components, capture proteins are deposited appropriately for detection and quantification of sample components present in low abundance (detection range: pg ml^{-1} to ng ml^{-1} range for many proteins).

Similarly, in a preferred embodiment in which the chips are used for the characterization of blood samples from animals, a chip contains a plurality of individual arrays (for example three arrays in a preferred example) as indicated above. Sample may then be diluted into a range of dilutions, consistent with the number of arrays available (Fig. 1). Dilutions of sample are generally performed with PBS or an equivalent buffer solution mimicking physiological pH and molarity. Typical dilutions for the example cited above comprise a "High-concentration" sample dilution, diluted 1:200; a "Medium-concentration" sample dilution, diluted 1:20; and a "Low-concentration" sample dilution, diluted 1:2. The results for a particular sample component (parameter) from these assays will indicate whether further dilutions and alteration of assay components are necessary to optimise detection and quantification. The internal control features and replication built into the array design, as described herein, indicate to the person skilled in the art the manner in which these factors should be adjusted in order to rapidly achieve adequate, reproducible, quantitative data.

The arrays of antibodies or proteins can be individually composed in accordance with the application (disease specific arrays, various body fluids of one individual *etc.*). Alternatively, if fewer parameters or such parameters lying in one concentration range have to be analysed in a larger set of samples, arrays of only one type may be printed onto the substrates. Processing these arrays with individual samples significantly decreases the time required for analysis of a set of parameters if compared to conventional ELISA format.

6. **Incubating the antibody arrays**

Generally, the chips of one experimental procedure are incubated, washed and blocked with a blocking solution (*vide infra*) a single, shared procedure even though they may carry individual arrays of different types. The reactions performed individually over the specific arrays are blocking, sample incubation, incubation with detection antibodies (if sandwich ELISA or equivalent format is used), and subsequent washes between the incubation steps. In a preferred embodiment the chips carrying only one array or several arrays with parameters detected in one sample dilution are incubated using the commercially available Coverplate™ systems (Shandon, Frankfurt, Germany). This system is generally compatible with liquid handling robots (in the preferred embodiment TECAN Genesis 150) and allows a fully automated incubation of the chips in a high throughput manner.

After printing the chips are placed into the Coverplate™ system, the glass surface is rinsed and blocked. In general, delivery of solutions containing proteins to be bound by the antibodies of the array may be preceded, followed, or accompanied by delivery of a blocking solution. A blocking solution contains protein or another moiety that will adhere to sites of non-specific binding on the array. For instance, solutions of bovine serum albumin (BSA), glycine, or powdered milk, or a combination of any of the above may be used as blocking solutions, such solutions preferably being buffered at physiological pH, for example with PBS. In a second preferred embodiment, three arrays requiring different sample dilutions are printed on one slide. Blocking, washing procedures, and detection is performed as described above. For sample incubation, between the three arrays lines of PaP-pen (MBT, Gießen, Germany) are drawn as a barrier, different sample dilutions or plasma of different animals are added separately onto the arrays and the chips (slides) incubated in a moist chamber.

In the preferred embodiment the rinsing is performed using 0.1 x PBS and the blocking in two steps using 3% BSA in PBS and 3 % BSA, 10 mM glycine in PBS. Following a wash step using PBS/Tween-20 the individual chips or arrays are incubated with the individual samples in an adjusted dilution. In the competitive immunoassay format, labelled standard proteins are added to the samples that are incubated on the

respective arrays. In a preferred embodiment of the invention the labelled standard proteins are biotin-labelled. After washing with PBS/Tween-20 the individual arrays of sandwich immunoassay format are incubated with the detection antibody solution, in the preferred embodiment consisting of 3% BSA and 10 mM glycine and detection antibodies in the appropriate dilution and combination. This step is omitted in the competitive immunoassay format. Preferably, in the competitive format, biotin-conjugated standard proteins are used, in the sandwich format biotin-conjugated detection antibodies. The corresponding preferred detection step performed in both formats comprises the addition to the arrays of streptavidin labelled with a detectable moiety, such as a chromophore or fluorophore, and appropriate incubation. In the most preferred embodiments of the invention, Cy™-conjugated streptavidin is employed.

After washing the slides extensively with PBS/Tween-20 and distilled water, slides are air dried and analysed using a laser scanning device (*e.g.* as commercially distributed by Axon Instruments Inc., USA; in the preferred embodiment of the invention a GenePix 4000A instrument is used). The scanning is performed over the entire chip independently of the number of arrays it may carry and the raw data are obtained *in toto* for all individual arrays. In a preferred embodiment of the invention intensities of each single spot detected at 635 nm (Cy5) and 532 nm (Cy3) are acquired together with background intensities. These raw data are used for further statistical evaluation of the respective experiment (Figures 3, 4 and 5).

7. Statistical data handling

The data sets that represent features in a population of samples are subjected to further analysis. In the preferred embodiment, the respective data rows are copied into Microsoft EXCEL, first background subtraction is performed and the data marked by error flags are removed. ("Error flags" are assigned to data that cannot be distinguished from background signal with a confidence level of at least 95%.) Cluster analysis is performed that is an alignment and sorting on the basis of similarity of data in their patterns (for example, by any of the correlation analyses that are well known in the art).

In addition, the minimum and maximum value of each group of replica (eleven spots) is removed and the average is calculated. Standard curves are calculated and the respective concentration of each parameter of all samples measured in one experiment is displayed as diagram. Standard curves are used as control for assay performance and can be used for calculation of concentrations (Fig. 4). Normalization of results of one experiment or between two independent experiments with respect to inter-chip and intra-chip variability can be performed by using reference antigen values (in this example gp41-values) of blocks of one array or different arrays on different chips.

8. Automated Data Processing

Applying the invention in an industrialized scale an automated procedure processing of the raw data in terms of normalization and standardization, calculation of distinct concentration out of signal intensities and quality control is a prerequisit. In the preferred embodiment of the invention a data mining pipeline was provided in which it is distinguished between entities for which distinct protein concentrations are of biological value and those for which a relative concentration in the sample is preferred. As outlined in Fig.7 after a background reduction the values from the slides incubated with protein standards are routinely used to calculate the standard curves, from which a distinct concentration of each individual sample can be deduced. For the determination of relative concentrations the median level of signal intensity for all values of a given entity in a large set of samples is determined and used for normalization of the samples and calculation of relative concentration. In those cases the standard curves, which are generated routinely are exclusively used for quality control. Routine quality control management is a central aspect to ensure maximum reliability and reproducibility of each individual assay. This is of particular importance when the assays are measured and evaluated in a fully automated fashion as described in the preferred embodiment of the invention. Automatic quality control is performed using the correlation coefficient of the standard curves to monitor assay performance and stability. Minimal signal intensities are defined to distinguish background noise from specific signals. Furthermore a parcer is provided in order to deliver the processed and quality checked data to the central data depository from which a more detailed analysis can be performed.

A preferred embodiment of the invention includes a protein micro-array for performing binding assays, said protein micro-array comprising molecules of at least one protein immobilized directly on a surface of a solid substrate in identified discrete regions (spots), wherein the arrangement of the discrete regions of protein on the substrate surface includes the following features:

(a) an unrelated binding assay is integrated into each individual block of each array, thereby providing controls for monitoring inter-chip and intra-chip variability; and

(b) the array comprises secondary binding proteins immobilized in identified discrete regions as an anchor row of the array, wherein said secondary binding proteins are labelled with one or more reporter molecules, thereby providing a control for variability of homogeneity of said discrete regions within the array. The binding assays that are performed may comprise immunoassays, and the unrelated binding assays integrated into each individual block of each array may comprise immunoassays. Moreover, the secondary binding proteins of said anchor row may comprise at least one secondary antibody.

A further preferred embodiment of the invention provides a protein micro-array of the invention wherein each array comprises a constant number of blocks, wherein each block comprises a constant number of rows of individual spots with a constant number of replicates, and wherein each replicate comprises:

(a) the primary (capture) binding proteins in at least two different concentrations; and

(b) a secondary (detection) binding protein labelled with a reporter molecule in at least two different concentrations. In such a protein micro-array the secondary (detection) binding protein may comprise a secondary antibody, and each replicate may further comprise detection antibodies of the corresponding parameter in at least two different concentrations.

The protein micro-array of the invention may further comprise secondary binding proteins of the anchor row which are labelled with a reporter molecule that is a fluorescence label, preferred fluorescence labels being indocarbocyanin and indodicarbocyanin. Furthermore, the immobilized protein of the protein micro-array of the invention may comprise an antibody, or at least one fragment of at least one antibody wherein said fragment is capable of binding to the epitope recognised by the antibody, such as a Fab fragment of at least one antibody. The immobilized protein

may comprise a biological receptor, an inter-cellular adhesion molecule, an antigen, or a recombinantly generated chimeric protein.

In preferred embodiments, the identified discrete regions arrayed on the substrate surface of the protein micro-array of the invention have an average diameter of at least 200 to 500 μm, average diameters of 50 to 200 μm, 10 to 50 μm, and less than 10 μm also being preferred. The number of these identified discrete regions arrayed on the substrate surface is 250 to 1000 in preferred embodiments, but may also be 1000 to 5000, 5000 to 25000, and greater than 25000.

The invention further includes a method of preparing a protein micro-array on a surface of a substrate wherein the buffer used to deposit the arrayed protein onto the surface comprises:

(a) an aliphatic electrically-charged detergent at a concentration between 0,01% (w/v) and 1,0% (w/v); and

(b) glycerol at a concentration between 1,0% (w/v) and 40% (w/v);

and wherein the pH of said buffer is between pH 5.5 and pH 9.5. In preferred embodiments the pH of said buffer is between pH 6.5 and pH 8.5; in more preferred embodiments it is between pH 7.0 and pH 8.0; even more preferred is for it to be between pH 7.2 and pH 7.6; and most preferred is for it to be between pH 7.2 and pH 7.6. The preferred concentration of said aliphatic electrically-charged detergent in said buffer is between 0,03% (w/v) and 0,3% (w/v); 0,07% (w/v) and 0,15% (w/v) being more preferred. It is preferable for the aliphatic electrically-charged detergent in said buffer to be negatively-charged, the most preferred detergent being sodium dodecyl sulphate. Preferred glycerol concentrations in said buffer are between 3% (w/v) and 10% (w/v), with 4,5% (w/v) and 5,5% (w/v) being the more preferred range.

The invention further encompasses a method for producing a protein micro-array on a surface of a substrate wherein said method comprises the following steps:

(a) preparing the surface to ensure the presence of covalently bound reactive groups before depositing and coupling protein onto the surface;

(b) depositing the arrayed protein onto said surface by the method of any of the invention, wherein the deposition of the protein is performed by an automated device; and

(c) allowing the resulting micro-array to dry on the surface of the substrate. The substrate may comprise a material selected from the group composed of: (a) glass; (b) plastic; (c) thin-layer covered slides and (d) membrane covered slides. In this method, said surface may exhibit covalently bound reactive groups selected from the group of: (a) aldehyde groups; and (b) carboxyl groups. In a preferred embodiment of such method, said surface is present on a glass substrate which exhibits covalently bound reactive groups that are aldehyde groups, and wherein said aldehyde groups are oxidised to generate activated carboxyl groups in step (a) of the process.

A further embodiment of the invention is a method (the analytical method) for estimating the concentration of at least one individual reactive molecular species within at least one individual by comparison with concentrations of standard proteins, wherein the accuracy of the estimation is quantitative, or at least semi-quantitative, wherein the analytical method employs the protein micro-array of the invention, and wherein the analytical method is a binding assay. The analytical method is preferably an immunoassay, in which the reactive molecular species is an immune reactive molecular species. The analytical method may comprise binding a polypeptide ligand onto the substrate of said micro-array and incubating with a protein solution that is either a test sample or a standard (reference) protein (*i.e.* direct format assay). The analytical method may alternatively comprise binding a capture protein onto the substrate of said micro-array and incubating with a ligand solution that is either a test sample or a standard ligand (*i.e.* 'sandwich' format assay). The analytical method may alternatively comprise binding a capture protein onto the substrate of said micro-array and incubating with a series of solutions comprising test sample pre-mixed with labelled, standard ligand at a range of defined concentrations (*i.e.* competitive format assay). The last-mentioned embodiment of this analytical method may comprise a labelled, standard ligand that is labelled with a reporter molecule comprising a label selected from the group consisting of radiochemical labels, fluorescence labels (preferably indocarbocyanin or indodicarbocyanin), and functional enzymes. The ligand in these analytical methods may be an antigen, and the capture protein may be an antibody.

The analytical method of the invention may comprise the use of a micro-array that provides a multiplicity of known concentrations for each capture protein employed

in the analytical method, and it may comprise the use of a plurality of micro-arrays that together provide a multiplicity of known concentrations for each capture protein employed in the method. It may further comprise contacting the micro-array with a multiplicity of sample dilutions; and it may further comprise incubating the micro-array with the sample, washing the micro-array to remove sample, and blocking the micro-array with a blocking solution, particularly where the method provides a competitive format assay. The secondary antibody or the reporter molecule may be labelled, biotin being a preferred label.

The analytical method of the invention may comprise contacting the micro-array with a labelled detection antibody directed toward the molecule captured by the capture protein, or the secondary antibody, followed by incubation of the micro-array with the detection antibody.

The analytical method of the invention may additionally comprise confocal scanning data acquisition, the analytical method may be performed by automated machinery, it may comprise statistical data handling of the quantitative data, including cluster analysis. The sample of the analytical method of the invention may be derived from a biological fluid, which may further be selected from whole blood, serum, plasma, urine, sinovial fluid, cerebrospinal fluid, a tissue extract, an organ extract, and a cell extract. The biological fluid may be of human origin. The analytical method may be used for the phenotypic characterisation of non-human animals, or it may be used for diagnostic screening where the sample is derived from humans, or from an organism selected from the following group: bacteria, yeast, insects, fish (particularly zebra fish), birds or mammals (particularly ungulates, but preferably rodents such as rats and mice.)

Embodiments of the invention include kits: a kit for producing the protein micro-array of the invention by the method of the invention wherein said kit comprises the substrate and the buffer used to deposit the arrayed protein onto the surface of said substrate; 'analytical' kits for performing the analytical method of the invention wherein said kit comprises a plurality of the protein micro-array of the invention, wherein the plurality provides said protein micro-array in which the capture protein is provided in at least two concentrations, resulting in an array selected from the group consisting of: an array for "High-concentration" sample detection and quantification;

an array for "Medium-concentration" sample detection and quantification; and an array for "Low-concentration" sample detection and quantification. Preferred 'analytical' kits are those in which the plurality of the protein micro-array of the invention is provided on an individual chip. The invention also encompasses an 'analytical' kit for performing the analytical method of the invention wherein said kit comprises the protein micro-array of the invention and at least one reagent selected from the group consisting of standard protein, standard ligand and labelled standard ligand.

The invention further encompasses use of the micro-array of the invention for simultaneous screening of multiple parameters in at least one individual sample, and use of the 'analytical' kit of the invention for simultaneous screening of multiple parameters in at least one individual sample, and preferably where the sample is derived from a biological fluid, which may be be selected from whole blood, serum, plasma, urine, sinovial fluid, cerebrospinal fluid, a tissue extract, an organ extract, and a cell extract. The biological fluid may be of human origin in said use.

A further embodiment of the invention is a use of the analytical method of the invention for the phenotypic characterisation of non-human animals, or the use of the analytical method for diagnostic screening where the sample is derived from humans, or from an organism selected from the following group: bacteria, yeast, insects, fish (particularly zebra fish), birds or mammals (particularly ungulates, but preferably rodents such as rats and mice.)

The scope of the invention will be illustrated further by reference to the following non-limiting examples. The following examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims:

Example 1. Fabrication of a two-dimensional antibody micro-array by contact printing

Primary (capture) antibodies were diluted to a final concentration of 125-500 ng/µl in printing buffer (Phosphate buffered saline, pH 7.4 with 0.05% SDS and 5%

glycerol). Biotin-conjugated detection antibodies were diluted to a final concentration of 2-8 ng/µl in printing buffer. As an anchor for easier scanning of the array Cy3-conjugated antibody (Dianova, Hamburg, Germany) was diluted 1:250 in printing buffer. For printing, 20-25 µl of each antibody containing solution was prepared and filled into a 384-well microtiter plate (Genetix, Hampshire,UK) at the appropriate position. Before loading into the microarrayer, the 384-well plate was centrifuged at 2000 x g for 5 minutes.

The split pins are removed from the tool holder and are cleaned by ultrasound in a detergent solution followed by water for five minutes each. One array consists of 11 x 11 spots per block, one block is printed by one split pin, spacing between the spots is set at 0.4 mm, and there may be up to eight arrays of the above dimension printed per substrate (2.5 x 7.5 cm^2). One array covers 1 x 1 cm^2. For the printing process, the first three slides are used for pre-printing to remove liquid from the outside of the pins. Pre-printing is performed 8 times per slide. For the printing process, each spot is composed of three strikes of the pin to the same surface area, before moving on to print the next spot. Between the pin-refills, a wash cycle is integrated. Pins are washed for 20 seconds in 70 % ethanol (v/v), 20 seconds in water and vacuum dried for 15 seconds. Dwell time of the pins in the wells of the respective 384-well micro-titre plate is set at 3 seconds.

After finishing the printing of the antibody solutions, the micro-arrays are allowed to dry (air-dried) for at least 3 hours or over night at room temperature to ensure optimal binding of the antibodies to the substrate surface. Fig. 2 provides an illustration of a preferred arrangement of the components of the microarray of the invention on the surface of the solid support.

Example 2. Competitive immunoassay using antibody micro-arrays on aldehyde-activated slides

Antibodies are arrayed as described in Example 1. One array composed of six parameters (see below) is printed per substrate. Capture antibodies as well as standard

proteins are purchased from BD Bioscience, Europe (Heidelberg, Germany). As solid support CSS-slides (CEL Associates Inc., USA) are used.

For competitive immunoassay, slides are placed into the Coverplate™ System (Shandon, Frankfurt, Germany) designed for histochemical staining. Slides are washed five times with 100 µl 0.1 x PBS, pH 7.4. Blocking is performed for 60 minutes in 3% BSA in PBS, pH 7.4 and 30 minutes in 3 % BSA, 10 mM glycine in PBS, pH 7.4. Slides are washed five times with PBS, 0.05 % Tween-20. Samples (dilution 1:200 in PBS) as well as standard protein mixture for standard curves are spiked with biotinylated standard protein mix (as indicated below) and added onto the arrays. Biotinylation of the standard proteins is performed using the Biotinylation Kit from Pierce (#21335ZZ Rockford, IL, USA), following the manufacturers recommendations.

For the standard curves, the standard protein concentrations indicated in the following table are used for each of the five immunoglobulins included in the array:

Slide #	Unlabelled standard protein concentration [ng/ml]
1	10000
2	2500
3	625
4	156.25
5	39.06
6	9.76
7	2.44
8	0.00

To each sample and standard protein dilution the following concentrations of biotin-conjugated standard proteins are added:

Parameter	Concentration [ng/ml]
IgA	3333
IgE	1667
IgG1	1000
IgG2a	1667
IgG3	10000
IgM	5000

Incubation of the samples on the antibody array is performed for two hours at room temperature. Five washes with PBS + 0.05% Tween-20 are performed and the arrays incubated with Cy5-conjugated streptavidin (Dianova, Hamburg, Germany) for 20 minutes. Five washes with PBS + 0.05% Tween-20 are performed, followed by extensive washes with distilled water. The slides are removed from the Coverplate™ system and air-dried.

Raw data acquisition is performed using the confocal laser scanner GenePix4000 (Axon Instruments Inc., Foster City, CA, USA) and the accompanying software (GenePix Pro Microarray acquisition and analysis software, Axon Instruments Inc., Foster City, CA, USA).

Example 3. Sandwich immunoassay using antibody micro-arrays

Antibodies are arrayed as described in Example 1. One array composed of the below listed parameters is printed per substrate. Commercially available antibodies are used in the array as follows:

Parameter	Capture Antibody	Detection Antibody	Standard Protein
IL-2	Biosource ARC0924	Biosource ARC0829	Biosource SD085
IL-4	BD 18190D	BD 18042D	BD 19231T
IL-5	BD 18050D	BD 18062D	BD 19241V
IL-10	BD 18140D	BD 18152D	BD 19281T
TNFα	BD 20030D	BD 23442D	BD 19321T
IFNγ	Biosource AMC4934	BD 18112D	BD 19301T
RANTES	R&D AF4781	R&D MAB478	R&D 478-MR-025
GM-CSF	Biosource AMC2014	Biosource AMC2929	Biosource PM2015

As substrate, CSS-slides (CEL Associates Inc., USA) are used. For sandwich immunoassay, antibody array carrying substrates are placed into the Coverplate™ System (Shandon, Frankfurt, Germany). Substrates are washed five times with 100 µl 0.1 x PBS, pH 7.4. Blocking is performed for 60 minutes in 3% BSA in PBS, pH 7.4 and 30 minutes in 3 % BSA, 10 mM glycine in PBS, pH 7.4. Following the blocking procedure substrates are washed five times with PBS + 0.05% Tween-20. The corresponding mixture of biotin-conjugated detection antibodies in the appropriate dilutions is added onto the substrates and incubated for two hours. Five washes with PBS + 0.05% Tween-20 are performed and the arrays incubated with Cy5-conjugated streptavidin for 20 minutes. Five washes with PBS + 0.05% Tween-20 are performed, followed by extensive washes with distilled water. The slides are removed from the coverplates and air-dried.

Raw data acquisition is performed using the confocal laser scanner GenePix4000 (Axon Instruments Inc., Foster City, CA, USA) and the accompanying soft ware (GenePix Pro Microarray acquisition and analysis software, Axon Instruments Inc., Foster City, CA, USA).

Example 4. Statistical procedure of data handling

Images of the antibody arrays are scanned using GenePix 4000 scanner and the micro-array acquisition and analysis software GenePix™Pro (Axon Instruments, Inc.). Of the acquired raw data, values of the 11 replica of one parameter for the respective wave length of the fluorescence dye used and the respective background values for each spot are copied into a Microsoft Exel work sheet. First the values marked with error flags by the analysis software are removed. In a following step, background subtraction is performed. Next, the minimal and maximal values are removed. The average and the standard deviation of the remaining values are calculated. If desired, a signal threshold can be defined so that if the intensities are too low, the whole experiment may be disregarded.

The average and standard deviation of each row of replica is then copied into a new worksheet and matched with the respective standard concentration if standard curves are drawn or with the respective sample number if plasma samples are evaluated. For standard curves the following tables are composed: (*see following page*)

ng/ml	IgG3 1:2	IgG3 1:4	IgG3 1:8
2000	842.75	420.285714	353
	142.587066	35.6731191	72.518963
400	1981.57143	709.25	431.4
	165.158366	86.8985451	59.8439638
80	2942	1016	542.571429
	246.686711	81.3224036	59.3685022
16	3076.85714	945	570.571429
	289.731156	209.621087	114.783647
0	2754.66667	1089	532.714286
	147.856236	142.687304	59.5307044

ng/ml	IgA 1:2	IgA 1:4	IgA 1:8
2000	443.166667	699	1464
	63.8354656	166.751312	184.92933
400	722.4	2072	3871.625
	174.754971	440.634542	506.655966
80	1488	3450	6409.16667
	439.567211	694.087026	774.513632
16	1536	3328.66667	5457.66667
	331.894059	517.551028	683.759802
0	1473.83333	2786.16667	3712.83333
	369.483649	434.50911	847.781438

Normalization of the data is performed in the next step, normalizing against values obtained for an unrelated antibody. In this example the unrelated antibody anti-gp41, a HIV trans-membrane protein, is used for normalization. The data acquired for anti-gp41 antibody, which is present in one concentration in each block of the arrays, are treated the same as the other parameters measured in the sample or standard protein mixture for standard curves. Each sample or standard protein mixture contains the same amount of gp41 protein, since it is added in before incubation. The average of all gp41 values of one experiment is calculated. To determine the normalization factor for the parameters of one block of an array this average is divided by the gp41 value of the respective block. The resulting factor is multiplied with each respective value of the

parameters in one block of an array. The resulting numbers are taken to calculate and draw standard curves.

Normalization performed with the values of the above data results in the following:

ng/ml	IgG3 1:2	IgG3 1:4	IgG3 1:8
2000	903.533268	450.598784	378.460093
400	1788.53732	640.158653	389.375316
80	3369.65001	1163.68607	621.439776
16	2690.73143	826.4086	498.968397
0	2476.46452	979.018586	478.913854

ng/ml	IgA 1:2	IgA 1:4	IgA 1:8
2000	475.130023	749.415312	1569.59087
400	652.02765	1870.15683	3494.47197
80	1704.29613	3951.49305	7340.80508
16	1343.24192	2910.94049	4772.76473
0	1324.98643	2504.78324	3337.86303

These resulting values are then used to draw standard curves in SIGMA PLOT 2001 (from SPSS Inc., Chicago IL, USA) as illustrated in Fig. 4. Sigmoid curve fitting with five parameters was employed for standard curves. The parameters were then used in the formula $x = x_0-b*((\ln(a/(value-y_0))^{\wedge}(1/c))-1$, as instructed for using SIGMA PLOT 2001, to calculate concentrations of the measured parameters in the respective samples.

Example 5. Processing of raw data during routine use

During the read-out of the arrays the primary data are obtained in arbitrary unit representing fluorescent signal intensities. In order to gain biological information from these data, an automated data processing and evaluation platform is part of the invention. After a background reduction two alternative processing procedures are available (see Fig. 7):

1. For Parameters for which distinct protein concentrations are of biological relevance, the standard curves are used to calculate distinct analyte concentration. Fig 8A shows a set of immunoglobulin data processed following that procedure. This type of analysis is preferred when analyte concentrations need to be compared to independent data of varying sources, for example independent experimental procedures or independently published data.

2. For the determination of relative concentrations the median level of signal intensity for all values of a given entity in a large set of samples is determined and used for normalization of the samples and calculation of relative concentration. In those cases the standard curves, which are generated routinely are exclusively used for quality control. Fig 8B displays a set immunoglobulin data (IgG1, IgM) processed processed accordingly. This method is preferred when the relative analyte concentration in a sample compared to the entire data set is of importance. This method is particularly preferred to identify outlyers with abnormally or extraordinarily high or low analyte levels.

Table 1: Components of different printing buffers.

Row number	Printing buffer
1	5 µg/ml BSA, 0,02% SDS, 10% glycerol, 1x PBS
2	40 % glycerol in PBS
3	5 % glycerol in PBS
4	PBS, 0.05% CHAPS
5	PBS, 0.05% CHAPS, 5% glycerol
6	PBS, 0.005% CHAPS
7	PBS, 0.005% CHAPS, 5% glycerol
8	PBS, 0.005% SDS
9	PBS, 0.005% SDS, 5 % glycerol
10	PBS, 0.0005% SDS
11	PBS, 0.0005% SDS, 5% glycerol
12	PBS, 0.005% SDS
13	PBS, 0.05% Triton-X-100
14	PBS, 0.05% Triton-X-100, 5% glycerol
15	PBS, 0.005% Triton-X-100
16	PBS, 0.005% Triton-X-100, 5% glycerol
17	PBS, 0.005% SDS, 5% DMSO
18	PBS, 5% DMSO
19	PBS, 0.5% CHAPS
20	PBS, 0.5% CHAPS, 5% glycerol
21	PBS, 0.5% Triton-X-100
22	PBS, 0.5% Triton-X-100, 5 % glycerol

5

References

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CLAIMS

1. A protein micro-array for performing binding assays, said protein micro-array comprising molecules of at least one immobilized protein, wherein said protein is immobilized directly on a surface of a solid substrate in identified discrete regions (spots), and wherein the arrangement of the discrete regions of protein on the substrate surface includes the following features:
   (a) an unrelated binding assay is integrated into each individual block of each array, thereby providing controls for monitoring inter-chip and intra-chip variability; and
   (b) the array comprises secondary binding proteins immobilized in identified discrete regions as an anchor row of the array, wherein said secondary binding proteins are labelled with one or more reporter molecules, thereby providing a control for variability of homogeneity of said discrete regions within the array.

2. The protein micro-array of claim 1 wherein the binding assays that are performed comprise immunoassays.

3. The protein micro-array of any of claims 1 or 2 wherein said unrelated binding assays integrated into each individual block of each array comprise immunoassays.

4. The protein micro-array of any of claims 1 to 3 wherein said secondary binding proteins of said anchor row comprise at least one secondary antibody.

5. The protein micro-array of any of claims 1 to 4 wherein each array comprises a constant number of blocks, wherein each block comprises a constant number of rows of individual spots with a constant number of replicates, and wherein each replicate comprises:
   (a) the primary (capture) binding proteins in at least two different concentrations; and

(b) a secondary (detection) binding protein labelled with a reporter molecule in at least two different concentrations.

6. The protein micro-array of claim 5 wherein said secondary (detection) binding protein comprises a secondary antibody.

7. The protein micro-array of any of claims 5 to 6 wherein each replicate further comprises detection antibodies of the corresponding parameter in at least two different concentrations.

8. The protein micro-array of any of claims 1 to 7 wherein said secondary binding proteins of said anchor row are labelled with a reporter molecule that is a fluorescence label.

9. The protein micro-array of any of claims 1 to 8 wherein said immobilized protein comprises an antibody.

10. The protein micro-array of any of claims 1 to 9 wherein said immobilized protein comprises at least one fragment of at least one antibody wherein said fragment is capable of binding to the epitope recognised by the antibody.

11. The protein micro-array of any of claims 1 to 10 wherein said immobilized protein comprises a peptide selected from the following group:

(a) Fab fragments of at least one antibody; and

(b) an inter-cellular adhesion molecule;

(c) a cellular receptor;

(d) a recombinantly generated chimeric protein; and

(e) an antigen.

12. The protein micro-array of any of claims 1 to 11 wherein the average diameter of said identified discrete regions arrayed on the substrate surface is selected from the group consisting of:

(a) diameter of 200 to 500 μm;

(b) diameter of 50 to 200 μm;

(c) diameter of 10 to 50 μm; and

(d) diameter of less than 10 μm.

13. The protein micro-array of any of claims 1 to 12 wherein the number of said identified discrete regions arrayed on the substrate surface is selected from the group consisting of:

(a) 250 to 1000;

(b) 1000 to 5000;

(c) 5000 to 25000; and

(d) greater than 25000.

14. A method of preparing the protein micro-array of any of claims 1 to 13, wherein said method comprises the step of depositing the immobilized protein onto said surface of a solid substrate with an automated device, and wherein the buffer used to deposit the arrayed protein onto the surface comprises:

(a) an aliphatic electrically-charged detergent at a concentration between 0.03% (w/v) and 0.3% (w/v); and

(b) glycerol at a concentration between 3.0% (w/v) and 10.0% (w/v);

and wherein the pH of said buffer is between pH 6.5 and pH 8.5.

15. A method of preparing the protein micro-array of any of claims 1 to 13, wherein said method comprises the following steps:

(a) preparing the surface to ensure the presence of covalently bound reactive groups before depositing and coupling protein onto the surface;

(b) depositing the arrayed protein onto said surface by the method of claim 14; and

(c) allowing the resulting micro-array to dry on the surface of the substrate.

16. The method of claim 15 wherein said substrate comprises a material selected from the group composed of: (a) glass; (b) plastic; (c) thin-layer covered slides and (d) membrane covered slides.

17. The method of any of claims 15 to 16 wherein said surface is present on a glass substrate which exhibits covalently bound reactive groups that are aldehyde groups, and wherein said aldehyde groups are oxidised to generate activated carboxyl groups in step (a) of the process.

18. A method for estimating the concentration of at least one individual reactive molecular species within at least one individual by comparison with concentrations of standard proteins, wherein the method employs the protein micro-array of any of claims 1 to 14, and wherein the method is a binding assay.

19. The method of claim 18 wherein the method is an immunoassay, and wherein the reactive molecular species is an immune reactive molecular species.

20. The method of any of claims 18 to 19 wherein a polypeptide ligand is bound onto said substrate of said micro-array and incubated with a protein solution selected from the group of:

(a) test sample; and

(b) standard protein.

21. The method of any of claims 18 to 19 wherein a capture protein is bound onto said substrate of said micro-array and incubated with a ligand solution selected from the group of:

(a) test sample; and

(b) standard ligand.

22. The method of any of claims 18 to 19 wherein a capture protein is bound onto said substrate of said micro-array and incubated with a series of solutions comprising test sample pre-mixed with labelled, standard ligand at a range of defined concentrations.

23. The method of claim 22 wherein said labelled, standard ligand is labelled with a reporter molecule comprising a label selected from the group consisting of:
(a) radiochemical labels;
(b) fluorescence labels; and
(c) functional enzymes.

24. The method of any of claims 21 to 23 wherein said capture protein is an antibody.

25. The method according to any of claims 18 to 24 wherein the method comprises a step selected from the following group:
(a) providing on a single micro-array a multiplicity of known concentrations for each capture protein employed in the method; and
(b) providing on a plurality of micro-arrays a multiplicity of known concentrations for each capture protein employed in the method.

26. The method according to claim 25 wherein the method comprises contacting the micro-array with the sample and incubating the micro-array with the sample; washing the micro-array to remove sample; and blocking the micro-array with a blocking solution.

27. The method according to any of claims 25 to 26 wherein the method additionally comprises, after incubation with sample, contacting the micro-array with a secondary antibody directed toward the molecule captured by the capture protein, and incubation of the micro-array with said secondary antibody.

28. The method of claim 27 wherein said secondary antibody is labelled.

29. The method according to any of claims 27 to 28 wherein the method comprises contacting the micro-array with a labelled detection antibody directed toward a molecule selected from the following group:

(a) the molecule captured by the capture protein;

(b) the secondary antibody;

and incubation of the micro-array with said detection antibody.

30. The method according to any of claims 25 to 29 wherein the method comprises contacting said micro-array with a multiplicity of sample dilutions.

31. The method according to any of claims 25 to 30 wherein the method additionally comprises confocal scanning data acquisition.

32. The method according to any of claims 25 to 31 wherein the method is performed by automated machinery and wherein the method additionally comprises statistical data handling of the quantitative data, particularly cluster analysis.

33. The method according to any of claims 18 to 32 wherein said sample is derived from a biological fluid selected from the group consisting of:

(a) whole blood;

(b) serum;

(c) plasma;

(d) urine;

(e) sinovial fluid;

(f) cerebrospinal fluid;

(g) a tissue extract;

(h) an organ extract; and

(i) a cell extract.

34. The method according to any of claims 18 to 33 for phenotypic characterisation or diagnostic screening of an organism selected from the following group:

(a) bacteria;

(b) yeast;

(c) insects;

(d) fish;

(e) birds;

(f) mammals.

35. The method according to any of claims 18 to 35 wherein said sample is of human origin.

36. The method of claim 34 wherein said organism is a rodent.

37. A kit for producing the protein micro-array of any of claims 1 to 13 by the method of any of claims 14 to 17 wherein said kit comprises the substrate and the buffer used to deposit the arrayed protein onto the surface of said substrate.

38. A kit for performing the method of any of claims 18 to 36 wherein said kit comprises a plurality of the protein micro-array of any of claims 1 to 13, wherein the plurality provides said protein micro-array in which the capture protein is provided in at least two concentrations, resulting in an array selected from the group consisting of:

(a) array for "High-concentration" sample detection and quantification;

(b) array for "Medium-concentration" sample detection and quantification; and

(c) array for "Low-concentration" sample detection and quantification.

39. The kit of claim 38 wherein said plurality of the protein micro-array of any of claims 1 to 14 is provided on an individual chip.

40. A kit for performing the method of any of claims 18 to 36 wherein said kit comprises the protein micro-array of any of claims 1 to 14 and at least one reagent selected from the group consisting of:

(a) standard protein;

(b) standard ligand;

(c) labelled standard ligand.

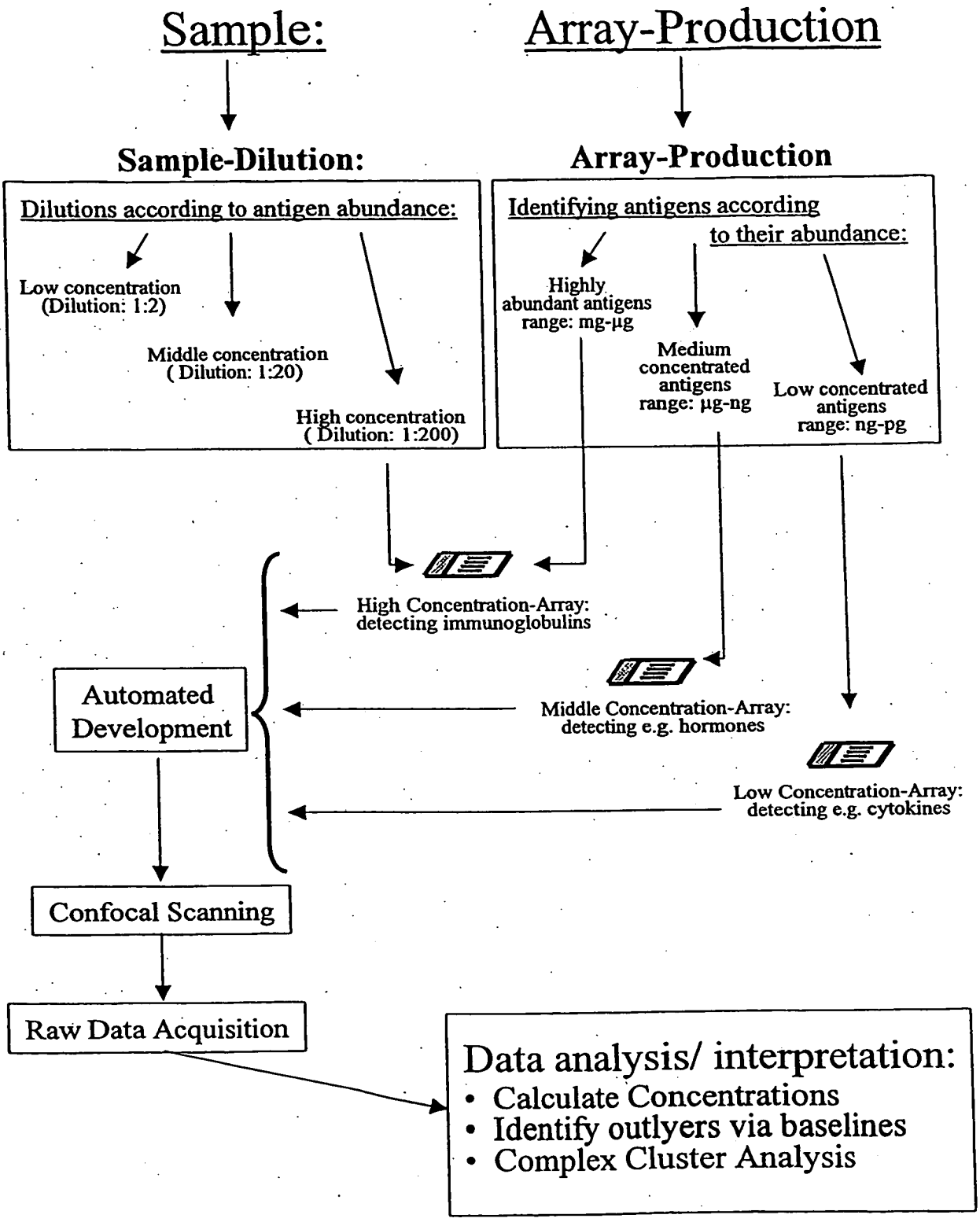

Sample:
Array-Production
Sample-Dilution:
Array-Production
Dilutions according to antigen abundance:
Low concentration (Dilution: 1:2)
Middle concentration (Dilution: 1:20)
High concentration (Dilution: 1:200)
Identifying antigens according to their abundance:
Highly abundant antigens range: mg-µg
Medium concentrated antigens range: µg-ng
Low concentrated antigens range: ng-pg
High Concentration-Array: detecting immunoglobulins
Middle Concentration-Array: detecting e.g. hormones
Low Concentration-Array: detecting e.g. cytokines
Automated Development
Confocal Scanning
Raw Data Acquisition
Data analysis/ interpretation:
• Calculate Concentrations
• Identify outlyers via baselines
• Complex Cluster Analysis

Fig. 1/8

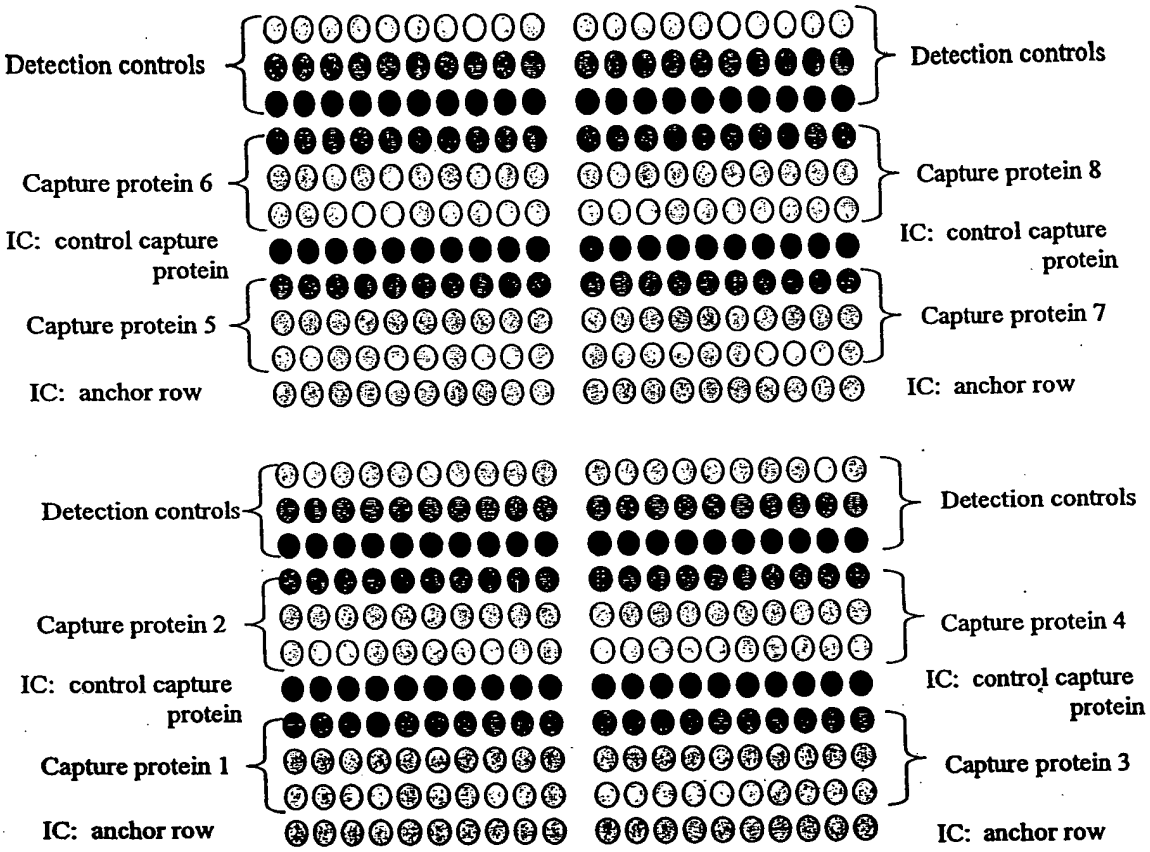
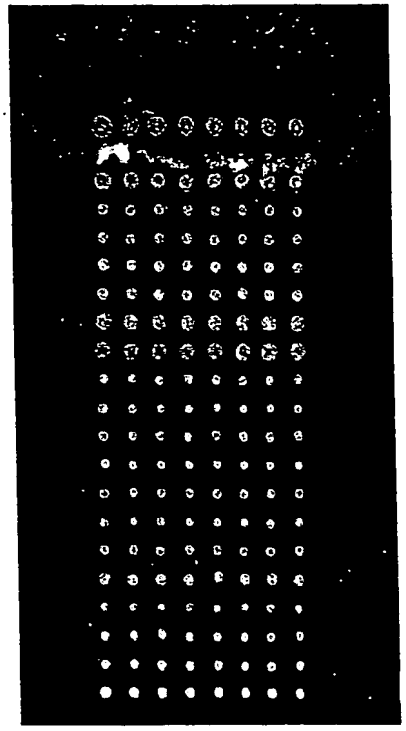

Detection controls
Capture protein 6
IC: control capture protein
Capture protein 5
IC: anchor row
Detection controls
Capture protein 8
IC: control capture protein
Capture protein 7
IC: anchor row
Detection controls
Capture protein 2
IC: control capture protein
Capture protein 1
IC: anchor row
Detection controls
Capture protein 4
IC: control capture protein
Capture protein 3
IC: anchor row

Fig. 2/8

A



B

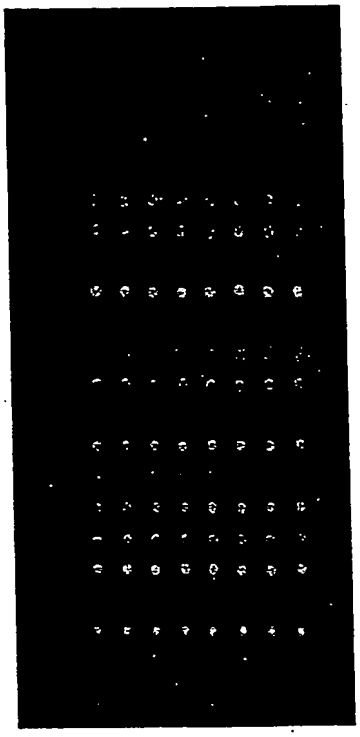
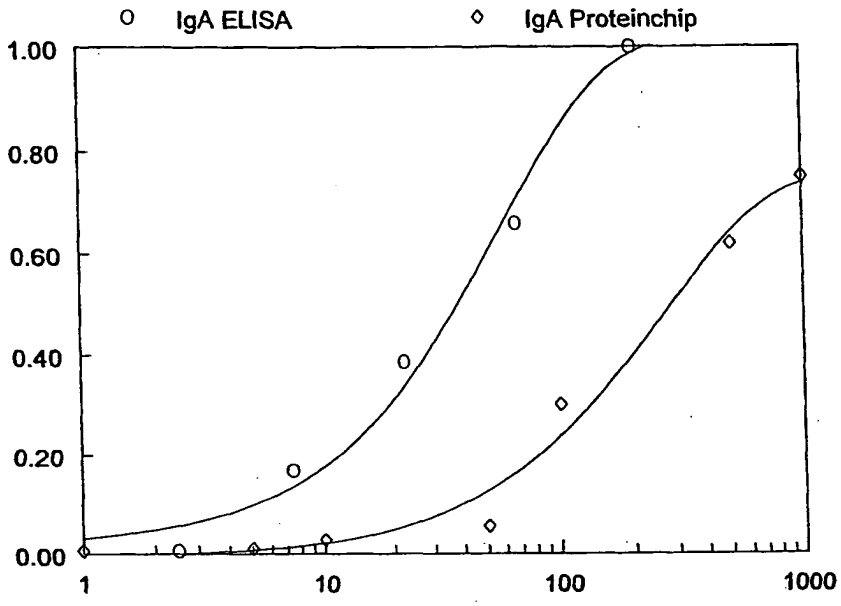


Fig. 3/8

A


IgA ELISA
IgA Proteinchip
1.00
0.80
0.60
0.40
0.20
0.00
1
10
100
1000

B

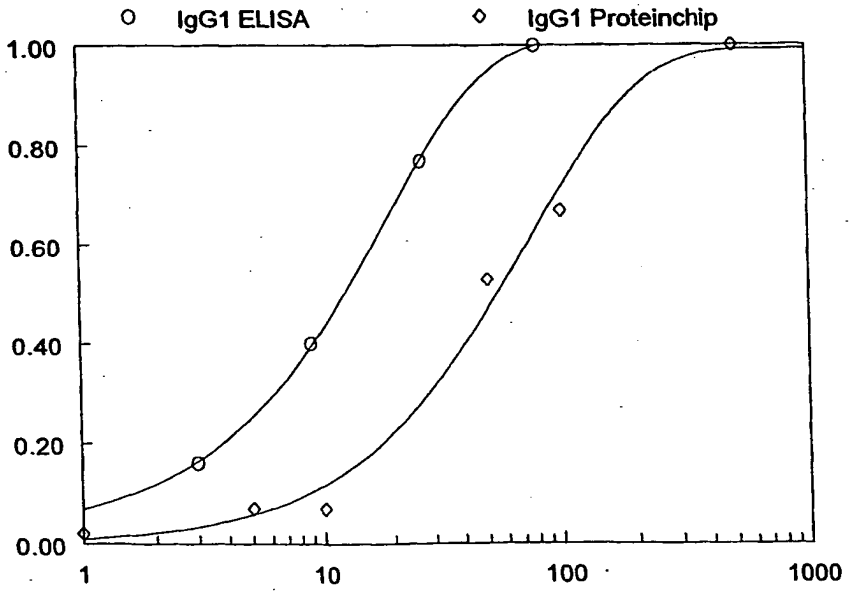
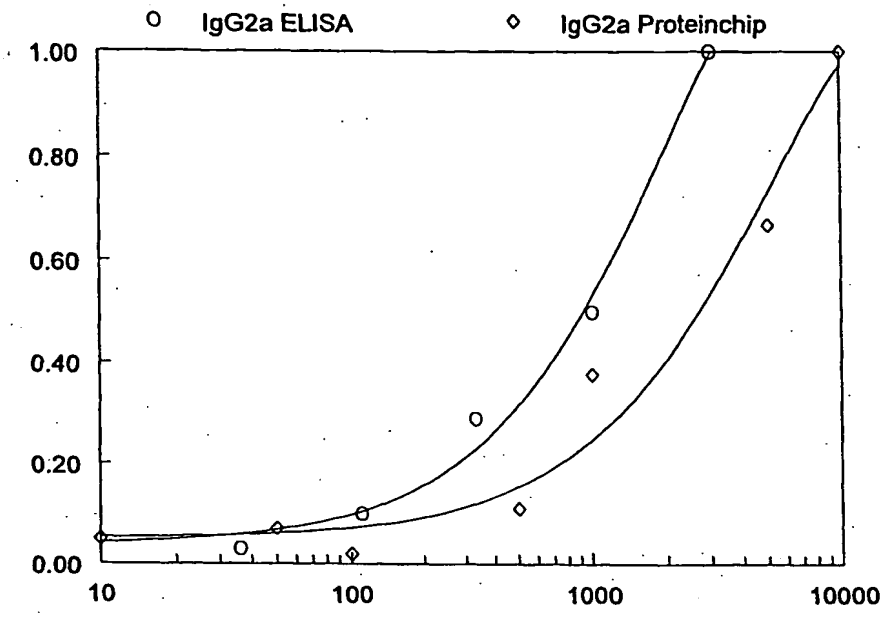

IgG1 ELISA
IgG1 Proteinchip
1.00
0.80
0.60
0.40
0.20
0.00
1
10
100
1000

Fig. 4/8-A, B

C


IgG2a ELISA
IgG2a Proteinchip
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0.80
0.60
0.40
0.20
0.00
10
100
1000
10000

D

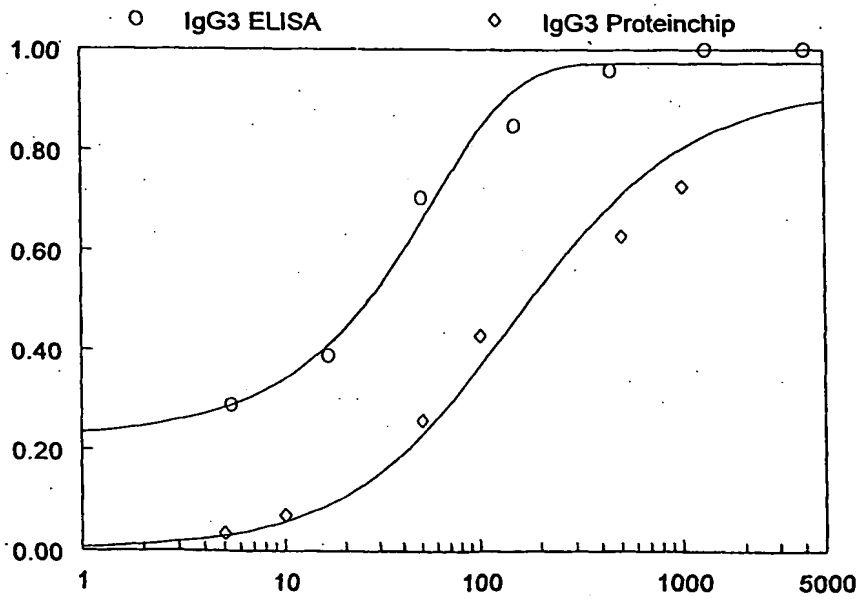
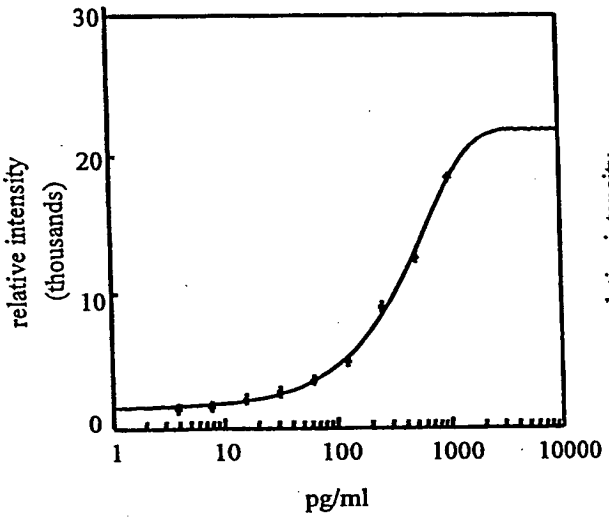
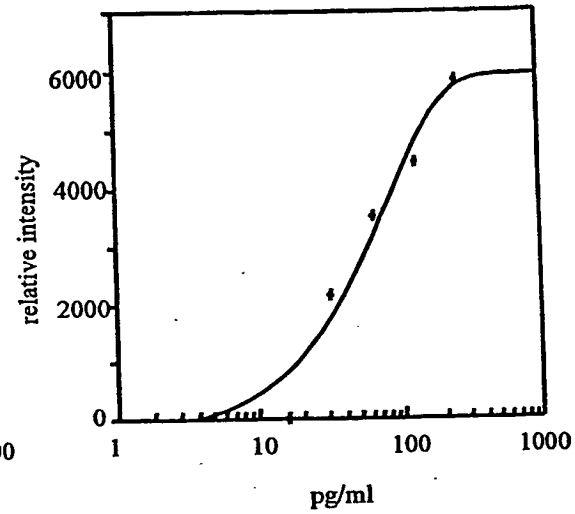

IgG3 ELISA
IgG3 Proteinchip
1.00
0.80
0.60
0.40
0.20
0.00
1
10
100
1000
5000

Fig. 4/8-C, D

A. Sandwich immunoassay

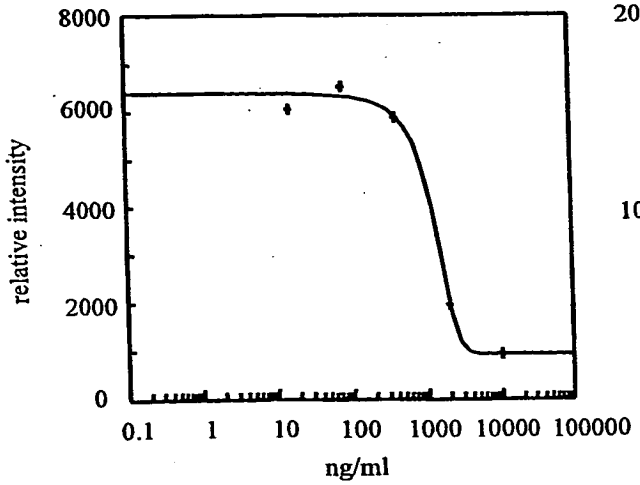


TNFα (r = 0.99)

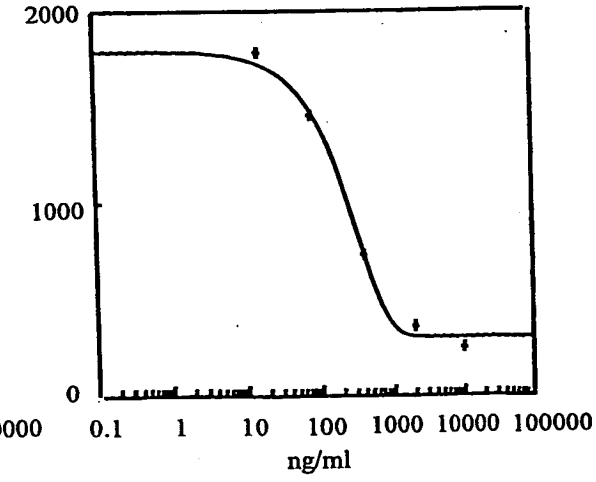


INF γ (r = 0.96)

B. Competitive immunoassay



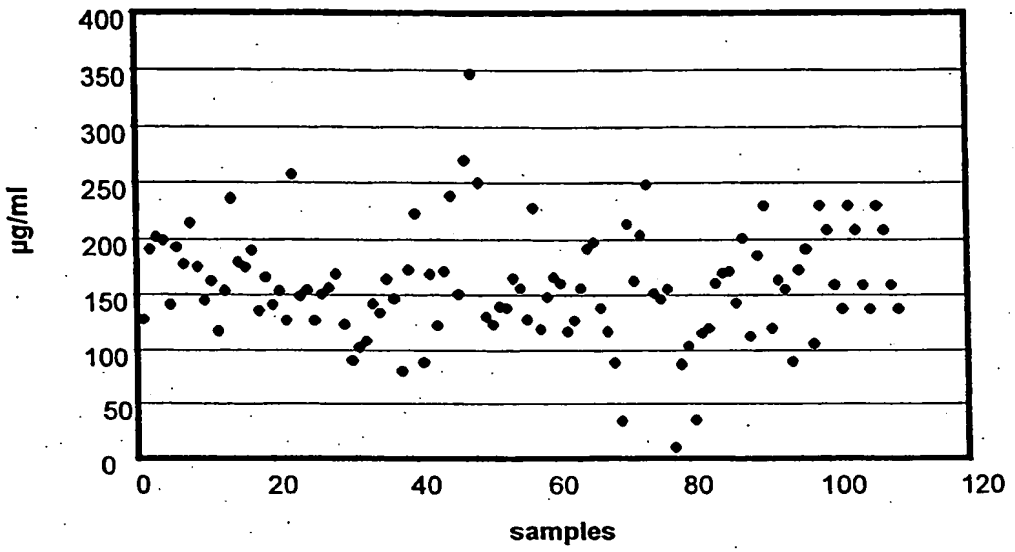
IgG2a (r =0.994)



IgG3 (r =0.995)

Fig. 5/8

A


IgA
400
350
300
250
200
150
100
50
0
µg/ml
0
20
40
60
80
100
120
samples

B

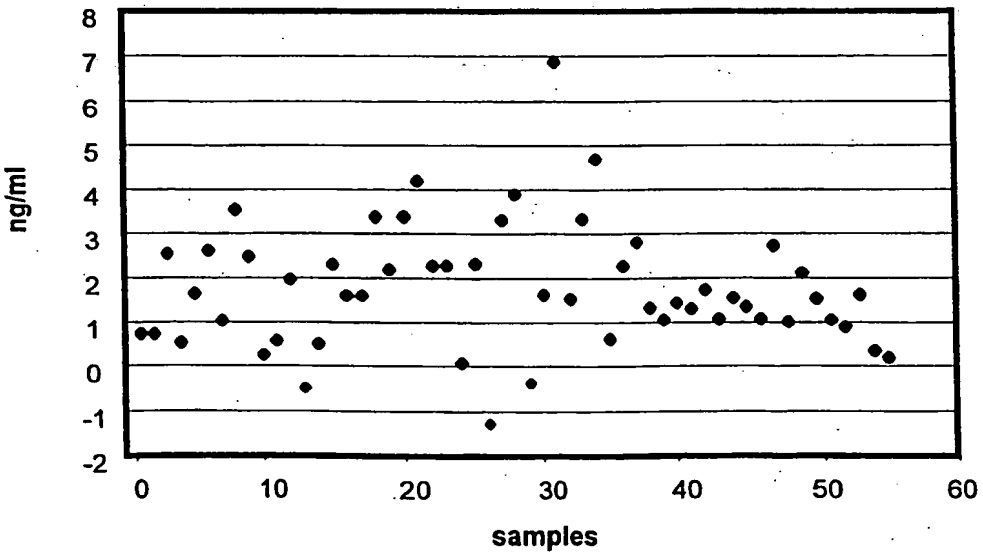

Luteinizing Hormone
8
7
6
5
4
3
2
1
0
-1
-2
ng/ml
0
10
20
30
40
50
60
samples

Fig. 6/8

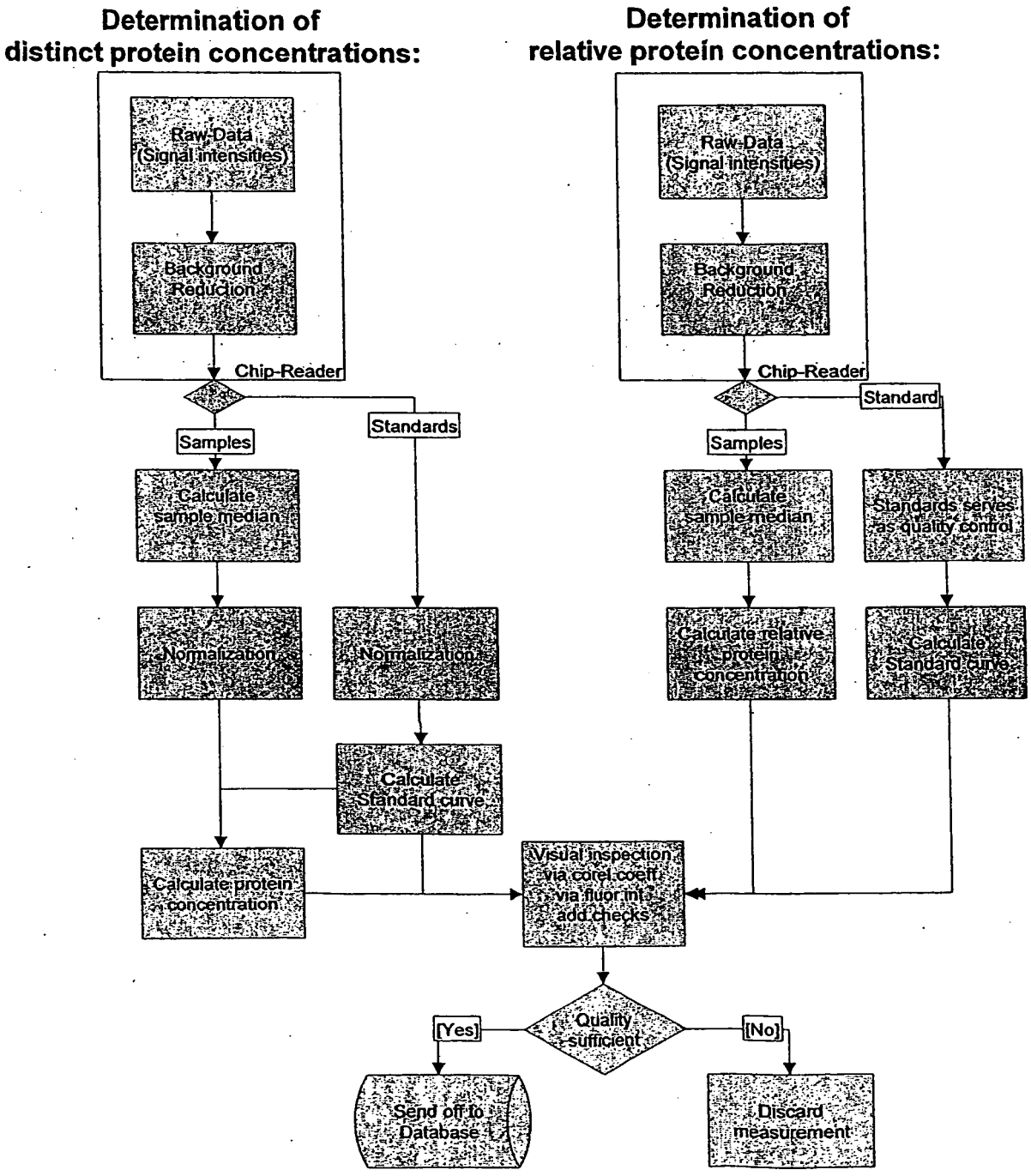

Determination of distinct protein concentrations:
Raw Data (Signal intensities)
Background Reduction
Chip-Reader
Samples
Standards
Calculate sample median
Normalization
Normalization
Calculate Standard curve
Calculate protein concentration
Determination of relative protein concentrations:
Raw Data (Signal intensities)
Background Reduction
Chip-Reader
Standard
Samples
Calculate sample median
Standards serves as quality control
Calculate relative protein concentration
Calculate Standard curve
Visual inspection via corel coeff. via fluor int. add checks
Quality sufficient
[Yes]
[No]
Send off to Database
Discard measurement

Fig. 7/8

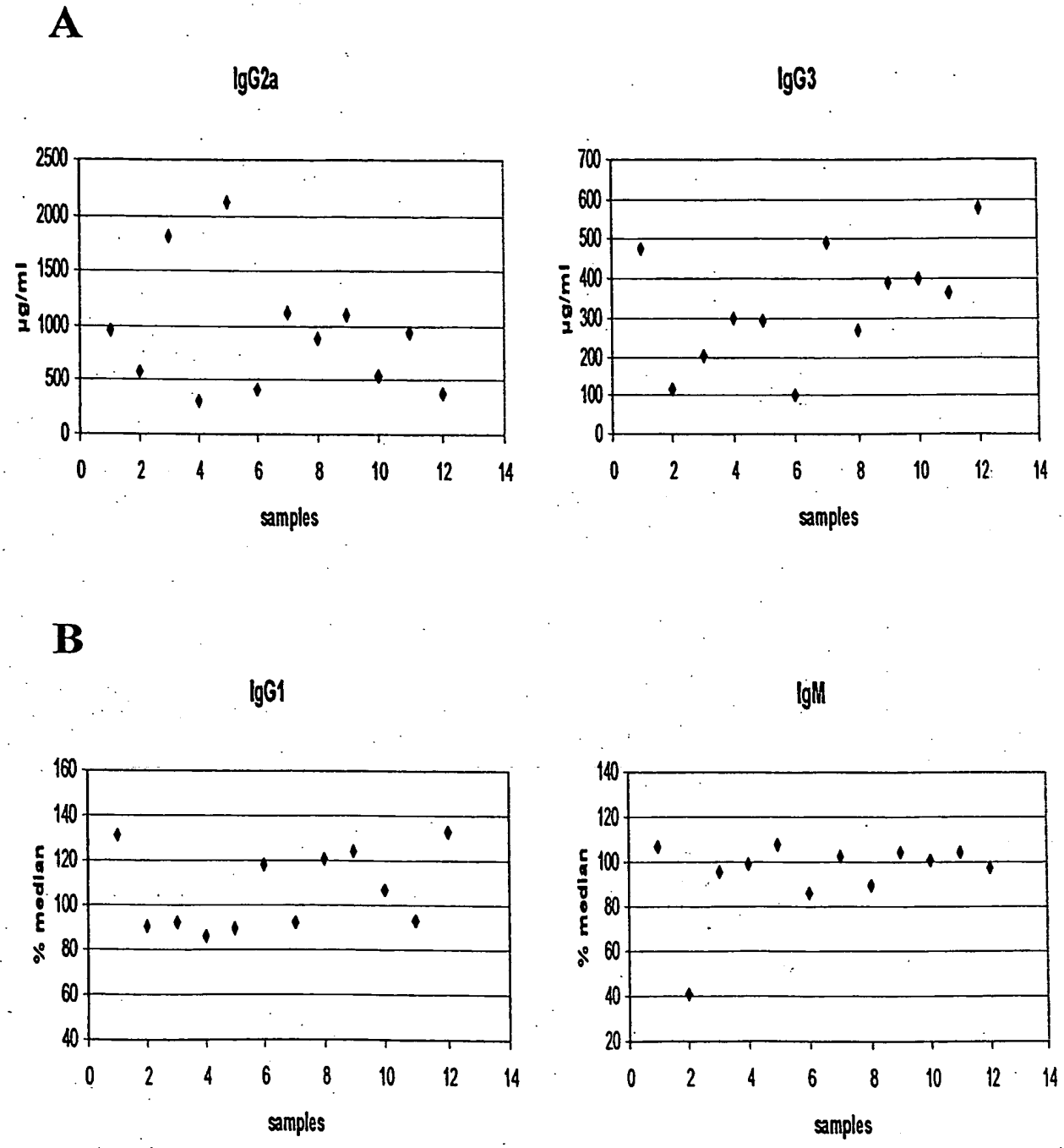

A
IgG2a
µg/ml
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14
samples
IgG3
µg/ml
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14
samples
B
IgG1
% median
160
140
120
100
80
60
40
0 2 4 6 8 10 12 14
samples
IgM
% median
140
120
100
80
60
40
20
0 2 4 6 8 10 12 14
samples

Fig. 8/8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/10538

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 G01N33/543 B01J19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 99 44062 A (THE UNITED STATES OF AMERICA) 2 September 1999 (1999-09-02) claims 1-20 ---	1-40
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier document but published on or after the international filing date
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O' document referring to an oral disclosure, use, exhibition or other means
'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2003	04/02/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreno de Vega, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/10538

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International Application No
PCT/EP 02/10538

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